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(71)(72) Applicants and Inventors: MARTIN, Daniel, S. [US/US]; 200 Haven Avenue, Apt. No. 5H, New York, NY 10033 (US). STOLFI, Robert, L. [US/US]; 9 Union Avenue, Harrison, NY 10528 (US). COLOFIORE, Joseph, R. [US/US]; 88 Jane Street, New York, NY 10014 (US). NORD, L., D. [US/US]; 4 Somer Drive, Somerville, NJ 08876 (US).

(74) Agent: BYRNE, Thomas, E.; Nixon & Vanderhye, 8th Floor, 1100 North Glebe Road, Arlington, VA 22201-4714 (US).

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(54) Title: CHEMOTHERAPEUTIC DRUG COMBINATIONS

(57) Abstract

The invention relates to certain compositions and combinations of ATP depletion compounds and apoptosis inducing agents. The invention also relates to methods of treating antineoplastic disease by administering such combinations.

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CHEMOTHERAPEUTIC DRUG COMBINATIONS

Field of the Invention

The present invention relates to compositions comprising compounds which deplete cellular energy. The invention also relates to combinations of such compounds with compounds which induce apoptosis. The invention also relates to the use of these compositions and combinations in the treatment of anti-neoplastic disease.

Background of the Invention

Adenosine triphosphate, ATP, is the key energy source in major metabolic processes such as biosynthesis, active transport and DNA repair. Consequently, if ATP production is inhibited, consumption of existing ATP will result in an energy deficiency that would adversely affect the functional and morphologic integrity of the cell.

Methods for selective impairment of energy metabolism have been proposed primarily involving induction of acidosis in tumors via sustained hyperglycemia, optionally in conjunction with acid-activated prodrugs of metabolic poisons (McCarty, Med Hypotheses 16:39-60 (1985)). This approach is contingent upon particular tumors having metabolic differences relative to normal tissues sufficient to produce acidosis, and is furthermore dependent on tumor mass being sufficiently large (and homogeneous) so that acidosis remains localized. The energy deficits thus produced are believed to be due to acidosis-induced reductions in tumor blood flow, which are furthermore dependent on the characteristics of vascularization of a particular tumor. This approach to impairing tumor energy metabolism is therefore not

universally, or even generally, applicable to treating a variety of forms of cancer.

Several compounds are known to impair cellular energy metabolism; these compounds are discussed below:

6-AN

The nicotinamide antagonist 6-aminonicotinamide (6-AN) (Johnson et al., Science 122:834 (1955)) is converted in vivo into the nicotinamide adenine dinucleotide (NAD) analogs, 6-ANAD and 6-ANADP. These competitive analogs of NAD and NADP cannot be reduced either chemically or enzymatically (Dietrich, Antineoplastic and Immunosuppressive Agents, ed. by A.C. Sartorelli and D.G. Johns (New York: Springer-Verlag), pp. 539-542 (1975), Dietrich et al., J. Biol. Chem. 233:964-968 (1958), and Dietrich et al, Cancer Res. 18:1272-1280 (1958)), and consequently act as potent inhibitors of NAD-dependent dehydrogenases utilized in glycolysis, in the oxidative portion of the pentose-phosphate pathway, and in mitochondrial oxidative phosphorylation (Dietrich, Antineoplastic and Immunosuppressive Agents, ed. by A.C. Sartorelli and D.G. Johns (New York: Springer-Verlag), pp. 539-542 (1975), Dietrich et al., J. Biol. Chem. 233:964-968 (1958), Dietrich et al , Cancer Res. 18:1272-1280 (1958), Woods et al. Biochem. Z. 338:381-392 (1963), Kaufman et al., J. Neurobiol. 5:391 (1974), Varnes, NCI Monographs No. 6, pp. 199-202 (1988), Ofori-Nkansah et al., Z. Krebsforsch 77:64-76 (1972), Ofori-Nkansah et al., Naunyn-Schmiedebergs Arch. Pharmacol. 272:156-168 (1972), Keller et al., Hoppe-Seyler's Z. Physiol. Chem. 353:1389-1400 (1972), Herken et al., Biochem. Biophys. Res. Comm. 36:93-100 (1969), Coper et al., Biochim. Biophys. Acta (Amst.) 82:167-170 (1964)). 6-AN has demonstrated preclinical anti-cancer activity (Martin et al., Cancer Res. 17:600-604 (1957), Hunting et al., Nature

Biochem. Pharmacol. 34:3999-4003 (1985)), but at tolerated doses, did not have antitumor efficacy as a single agent in humans (Herter et al., Cancer Research 21:31-37 (1961)).

MMPR

The adenosine analog 6-methylmercaptopurine riboside (MMPR) has been shown to result in ATP and GTP depletion, inhibition of macromolecular synthesis, and inhibition of tumor growth (Elion, Fed. Proc. 26:898-903 (1967), Elion, J. Am. Chem. Soc. 74:411-414 (1952), Shantz et al., Cancer Res. 33:2867-2871 (1973), Woods et al., Eur. J. Cancer 14:765-770 (1978), Warnick et al., Cancer Res. 33:1711-1715 (1973), Nelson et al., Cancer Res. 32:2034-2041 (1972)). In addition, the biosynthesis of NAD may be inhibited by MMPR because NAD is synthesized in the cell from nicotinamide mononucleotide (NMN) and ATP by an enzyme (NMN adenyltransferase) that is inhibited by thio-ITP (Atkinson et al., Nature 192:946-948 (1961)).

The 6-AN and MMPR Combination

MMPR and 6-AN are synergistic if the timing of their administration is appropriate, because the lowering of NAD levels by MMPR favors the competition of 6-ANAD with NAD, and thereby enhances the magnitude of ATP depletion that is achieved by either drug alone. The combination of 6-AN and MMPR produces regressions of advanced murine breast tumors which cannot be obtained with either drug alone (Martin, Mt. Sinai J. Med. 52:426-434 (1985)). In addition to its antipurine action, MMPR, in high dosage, is reported to decrease pyrimidine ribonucleotide concentrations *in vivo* (Woods et al., Eur. J. Cancer 14:765-770 (1978), Grindey et al., Cancer Res. 36:379-383 (1976)).

PALA

The pyrimidine antagonist N-(phosphonacetyl)-L-aspartic acid (PALA), in low non-toxic dosage, can lower pyrimidine levels in vivo selectively in certain tumors (Martin et al., Cancer Res. 43:2317-2321 (1983)).

The Triple Combination

The triple combination of PALA + MMPR + 6-AN was evaluated against advanced solid tumors in mice (Martin, Biochemical modulation -- Perspectives and Objectives. In: New Avenues in Developmental Cancer Chemotherapy. Ed. by Kenneth R. Harrap, London, England (1987)). Preliminary biochemical data (Martin, Metabolism and Action of Anti-cancer drugs. Ed. by Garth Powis and Russell A. Prough (London: Taylor & Francis), pp. 91-140 (1987)), demonstrated substantial depression at 24 and 48 hours of NAD levels, and of the four ribonucleoside triphosphates of purines and pyrimidines, including ATP. The three drug combination produced a marked antitumor effect which was not obtained with any of the individual agents or any combination of two of the agents (Martin, Mt. Sinai J. Med. 52:426-434 (1985), Martin, Biochemical modulation -- Perspectives and Objectives. In: New Avenues in Developmental Cancer Chemotherapy. Ed. by Kenneth R. Harrap, London, England (1987)).

Objects of the Invention

It is an object of the invention to provide compositions which deplete cellular energy.

It is a further object of the invention to provide drug combinations which are useful in treating antineoplastic disease.

Summary of the Invention

The subject invention relates to chemotherapeutic drug combinations and their use in the treatment of antineoplastic disease. Such drug combinations comprise: a) cellular energy depletion compounds, and b) at least one apoptosis inducing agent.

Advantageously, the cellular energy depletion compounds comprise:

1) an inhibitor of purine nucleotide biosynthesis,
2) a nicotinamide antagonist, and optionally
3) an inhibitor of pyrimidine nucleotide biosynthesis.

Brief Description of the Drawings

Figure 1 shows changes in PCr/Pi and NTP/Pi after treatment with the triple drug combination PALA, MMPR, and 6-AN. The change in PCr/Pi and NTP/Pi at 10 hours is statistically significant (P less than 0.01, p less than 0.02). The change in NTP/Pi between the 10 and 24 hour measurement is not significant. Pretreatment values were determined prior to the administration of $PALA_{100}$ and appear on the y intercept. At Time "0" $MMPR_{150}$ + 6- AN_{10} were administered. n = 7 for each time point.

Figure 2 shows results obtained in Example 2. CD8F1 mice bearing spontaneous, autochthonous breast tumors received three courses of treatment on a q 10-11 day schedule with Adria alone at 11 mg/kg, or with Adria at 6 mg/kg administered 2½ hrs after PALA + MMPR + 6-AN (PALA at 100 mg/kg 17 hrs before MMPR at 150 mg/kg plus 6-AN at 10 mg/kg), or with the same regimen of PALA + MMPR + 6-AN without Adria. Tumors averaged 304 mgs at initiation of therapy.

Detailed Description of the Invention

The subject invention relates to drug combinations comprising cellular energy depletion compounds and at least one apoptosis inducing agent. The invention also relates to the use of such drug combinations in the treatment of antineoplastic disease.

Apoptosis Inducing Agents

In the subject invention, apoptosis inducing agents are used in conjunction with cellular energy depletion compounds.

In the physiological context, apoptosis is a process by which cells are removed from embryonic and developing somatic tissues, and has been implicated in terminal differentiation of myeloid cells, and in hormone-dependent tissue atrophy. It has also been documented in the cytoxic T-cell killing of tumor cells, and in tumor regression (Wyllie et al., Int. Rev. Cytology 68:251-306 (1980)). Cell death via necrosis is characterized by cell swelling, chromatin flocculation and disruption of cell integrity followed by cell lysis (Wyllie et al., Int. Rev. Cytology 68:251-306 (1980)).

Cell death induced by anti-cancer agents may take the form of both apoptosis and necrosis. Apoptotic cell death may be more apparent at low levels of these agents, while at high levels (which therefore pose greater risk of toxic side effects) necrosis may occur due to severe metabolic insult. Hence, cells which are not killed directly, but merely injured by anti-cancer agents, may activate a genetically programmed "suicide" mechanism (Lennon et al., Cell Prolif. 24:203-214 (1991)).

A large number of agents have been demonstrated to induce apoptosis (apoptosis inducing agents), including:

Antimetabolites

•methotrexate (Barry et al., Biochem. Pharmacology 40:2353-2362 (1990) and Marks et al., Biochem. Pharmacol. 42:1859-1867 (1990)),

•5-fluorodeoxyuridine (Barry et al., Biochem. Pharmacology 40:2353-2362 (1990) and Kyprianou et al., Biochem. Biophys. Res. Communications 165:73-81 (1989)),

•5-fluorouracil (FUra) (Barry et al., Biochem. Pharmacology 40:2353-2362 (1990) and Kyprianou et al., Biochem. Biophys. Res. Communications 165:73-81 (1989)),

•1-B-D-arabinofuranosyl-cytosine (Gunj et al., OOOOCancer Res. 51:741-743 (1991)),

•puromycin (Kaufmann, Cancer Res. 49:5870-5878 (1989)),

•trifluorothymidine (Kyprianou et al., Biochem. Biophys. Res. Communications 165:73-81 (1989)),

DNA Damaging Agents

•cisplatin (Barry et al., Biochem. Pharmacology 40:2353-2362 (1990)),

•etoposide (Barry et al., Biochem. Pharmacology 40:2353-2362 (1990), Kaufmann, Cancer Res. 49:5870-5878 (1989), Tanizawa et al., Exp. Cell. Res. 185:237-246 (1989) and Marks et al., Biochem. Pharmacology 42:1859-1867 (1990)),

•camptothecin (Kaufmann, Cancer Res. 49:5870-5878 (1989)),

•cytoxan (Kaufmann, Cancer Res. 49:5870-5878 (1989) and Marks et al., Biochem. Pharmacology 42:1859-1867 (1990)),

•adriamycin (adria) (Marks et al., Biochem. Pharmacology 42:1859-1867 (1990)),

•teniposide (Kaufmann, Cancer Res. 49:5870-5878 (1989)),

•podophyllotoxin (Kaufmann, Cancer Res. 49:5870-5878 (1989)),

•aphidocolin (Barry et al., Biochem. Pharmacology 40:2353-2362 (1990) and Martin et al., Cell Tissue Kinet. 23:545-559 (1990)),

•N-methyl-N'-nitro- N'mitrosoguanidine (Barry et al., Biochem. Pharmacology 40:2353-2362 (1990)),

•nitrogen mustard (O'Connor et al., Cancer Res. 51:6550-6557 (1991)),

•bleomycin (Kuo et al., Nature 271:83-84 (1978)),

•1,3-bis(2-chloroethyl)-a-nitrosourea (BCNU, Berger et al., Cancer Res. 42:4382-4386 (1982)),

•methyl glyoxal-bis-(guanylhydrazone) (MGBG, Brune et al., Exp. Cell Res. 195(2):323-329 (1991)),

•radiotherapy (Warters, Cancer Res. 52:883-890 (1992)).

Microtubule Modifiers

•colcemid (Kaufmann, Cancer Res. 49:5870-5878 (1989)),

•vincristine (Martin et al., Cell Tissue Kinet. 23:545-559 (1990)),

•taxol (Martin et al., Cell Tissue Kinet. 23:545-559 (1990) and Lennon et al., Cell Prolif. 24:203-214 (1991))

•taxotere

Hormones

•dexamethasone (Barry et al., Biochem. Pharmacology, 40:2353-2362 (1990)),

•retinoic acid (Piacentini et al., Eur. J. Cell Biol., 54:246-254 (1991)),

•purinergic P2 receptor agonists (Trepel et al., WO Pat. Appl. # 9116056-A)

•somatostatin analogs (Pagliacci et al., Endocrinology, 129:2555-2562 (1991))

•luteinizing hormone releasing factor analogs (Szende et al., Cancer Res., 50:3716-3721 (1990))

•estrogen ablation (Kyprianou et al., Cancer Res., 51:162-166 (1991))

•tumor necrosis factor (Piguet et al., Am J. Pathol., 136:103-110 (1990))

Miscellaneous

•tumoricidal antibodies capable of inducing apoptosis (Krammer, Pat. WO9110448-A)

- cytotoxic T-cells (Ucker, Nature, 327:62-63 (1987))
- sodium azide
- gossypol
- lonidamine
- rhodamine 123

The significance of the preceding list is that these different agents are all capable of inducing the same final common mechanism of cancer cell death, regardless of the diverse biochemical lesions initiated by each individual agent. Current information indicates that the apoptotic biochemical cascade can be activated at different points in different cell types (Duvall et al., Immunology Today 7(4):115-119 (1989)).

The invention provides drug combinations and methods for impairing cellular energy and nucleotide metabolism, and thereby dramatically increasing the antitumor efficacy of a wide variety of apoptosis-inducing antineoplastic agents, without a corresponding increase in host toxicity. Since apoptosis is itself an energy-requiring process (Cotter et al., Anticancer Res., 10:1153-1159 (1990)), this result is unexpected.

The triple drug combination, PALA-MMPR-6-AN, as well as all apoptosis-inducing anti-cancer agents, have ATP depletion as a basic biochemical lesion, and complement each other on this basis. In addition, the PALA-MMPR-6-AN combination produces a level of wide biochemical damage in cancer cells that complements, and is complemented by, the apoptosis-inducing biochemical effects of DNA-damaging agents, and thereby result in enhanced kill of tumor cells. The in vivo addition of 5-fluorouracil, adriamycin, taxol, radiotherapy, mitomycin C,

cis-platinum, cytoxan, phenylalanine mustard, and etoposide with the PALA-MMPR-6-AN triple combination demonstrate greater anticancer activity than that observed with the individual drugs alone at maximum tolerated dose (MTD) or with the PALA-MMPR-6-AN combination alone.

Cellular Energy Depletion Compounds

Intracellular ATP has been negelected as a primary target for cancer chemotherapy because ATP has long been considered too important a source of energy for all cells to warrant the expectation that primarily directed anti-ATP chemotherapy could be selective for cancer cells and safe for host tissues.

The subject application documents novel compositions and methods for impairing tumor cell energy metabolism, with striking therapeutic activity, a safe therapeutic index, and an associated depletion of ATP levels in the in vivo treated tumors. When administered in conjunction with agents which trigger apoptosis (programmed cell death) in tumors, the energy-depleting compositions of the invention produce therapeutic activity against spontaneous tumors in vivo substantially greater than can be obtained by either energy depletion compositions or apoptosis-inducing agents alone.

Although glycolysis provides some of the ATP needed by the cell, the major generation of ATP occurs during oxidative phosphorylation in the mitochondria of mammalian cells as electrons are transferred from reduced NAD (NADH) to O_2 by a series of electron carriers. NADH is reconverted to NAD with concomitant conversion of ADP to ATP. In addition to their central role in energy metabolism, NAD^+ and $NADP^+$ (nicotinamide adenine dinucleotide phosphate), are oxidizing agents which function as coenzymes in critical biochemical reactions.

Disruptions in NAD synthesis and metabolism has profoundly adverse effects on cellular integrity because of the central role played by these coenzymes in intermediary metabolism including the generation of ATP from ADP. A limitation of adenine or NAD, or both, are keys to ATP depletion. In the subject invention, these metabolites are targets for chemotherapy designed to result in depletion of cellular levels of ATP.

The cellular energy depletion compounds of the invention are typically used in combination and typically include:

1) an inhibitor of purine nucleotide biosynthesis,
2) a nicotinamide antagonist, and optionally
3) an inhibitor of pyrimidine nucleotide biosynthesis.

HPLC and NMR measurements of biochemical changes resulting from treatment with PALA (an inhibitor of pyrimidine biosynthesis) + MMPR (an inhibitor of purine biosynthesis) + 6-AN (a nicotinamide antagonist) indicate a severe depletion of cellular energy levels in the treated tumors. The PALA-MMPR-6-AN-induced reduction in all four individual ribonucleoside pools (Martin, Metabolism and Action of Anti-cancer drugs. Ed. by Garth Powis and Russell A. Prough (London: Taylor & Francis), pp. 91-140 (1987)), which generally correlates with a reduction in the corresponding deoxyribonucleoside triphosphate pools (Hunting et al., Can. J. Biochem. 59:821-829 (1981)), appears to not only deplete cellular energy sources and to inhibit DNA synthesis, but to inhibit the potential for DNA repair as well. Because the DNA damage produced by many chemotherapeutic drugs is subject to repair, the cytotoxic activity of these DNA-damaging drugs is increased when the DNA repair potential of the cell is decreased. A 3-drug combination,

PALA-MMPR-6-AN, augments the antitumor activity of DNA-damaging drugs by virtue of its ability to deplete both the deoxyribonucleotides, and the energy source required for the DNA repair processes.

Inhibitors of Purine Nucleotide Biosynthesis

Inhibitors of purine nucleotide biosynthesis of the invention include the following:

a) Direct Inhibitors

6-methylmercaptopurine riboside (MMPR)
6-mercaptopurine
Thioguanine
Thiamiprine
Tiazofurin
Azaserine
6-diazo-5-oxo-L-norleucine
Acivicin

b) Folate Antagonists

Methotrexate
Trimetrexate
Pteropterin
Denopterin
Didiazotetrahydrofolate (DDATHF)

Advantageous inhibitors of purine nucleotide biosynthesis are MMPR and folate antagonists which are relatively selective inhibitors of the enzyme glycinamide ribonucleotide transformylase, e.g. DDATHF or DACTHF.

Nicotinamide Antagonists

Nicotinamide antagonists of the invention include the following:

6-aminonicotinamide (6-AN)

Thionicotinamide
2-amino-1,3,4-thiadiazole
2-ethylamino-1,3,4-thiadiazole
6-aminonicotinic acid
5-methylnicotinamide
3-acetylpyridine

Inhibitors of Pyrimidine Biosynthesis

Inhibitors of pyrimidine nucleotide biosynthesis of the invention include the following:

N-(phosphonoacetyl)-L-aspartic acid (PALA)
6-azauridine
Triacetyl-6-azauridine
Pyrazofuran
Brequinar
Acivicin

* * *

While not wishing to be bound by theory, the following is offered as an explanation of the mechanism of the combinations of the invention. The triple combination of an inhibitor of purine biosynthesis, a nicotinamide antagonist and an inhibitor of pyrimidine biosynthesis (e.g., PALA + MMPR + 6-AN) was designed to produce a mutually reinforcing blockade on pyrimidine and purine de novo biosynthesis, as well as a specific attack on NAD metabolism, that in toto result primarily in a damaging depletion of high energy nucleoside triphosphates, particularly ATP.

PALA is an inhibitor of de novo pyrimidine biosynthesis (Collins et al., J. Biol. Chem. 246:6599-6605 (1971), Johnson et al., Cancer Res. 36:2720-2725 (1976)). PALA has been found to be non-toxic to the hemopoietic

systems in mice (Johnson et al., Cancer Res. 36:2720-2725 (1976), Harrison et al., Cancer Chemother. Pharmacol. 2:183-187 (1978)), and when administered at a low dose (100 mg/kg) exerts selective action in CD8F1 breast tumor tissue, but not in the intestinal tissue of the host (Martin et al., Cancer Res. 43:2317-2321 (1983)).

The antitumor toxicity of high dose MMPR as a single agent is known to be associated with a general depletion of purine nucleotides that results in the inhibition of macromolecular synthesis and tumor growth (Nelson et al., Cancer Res. 32:2034-2041 (1972), Scholar et al., Cancer Res. 32:259-269 (1972), Warnick et al., Cancer Res. 33:1711-1715 (1973), and Woods et al, Eur. J. Cancer 14:765-770 (1978)). This thiopurine-induced decrease in tumor ATP levels has been implicated previously as the causal mechanism in the therapeutic effect produced in tumors (Atkinson, Regulation of energy metabolism: Exploitable molecular mechanisms and neoplasia. The University of Texas M.D. Anderson Hospital and Tumor Institute at Houston, 22nd Annual Symposium on Fundamental Cancer Research, 1968. (Baltimore: Williams and Wilkins), 397-413 (1969)).

The earliest reported studies emphasizing ATP depletion as a chemotherapeutic approach in vivo employed 6-AN as a single agent to lower ATP levels in the tumor and thereby produced effective antitumor activity (Dietrich et al., Cancer Res. 18:1272-1280 (1958) and Martin et al., Cancer Res. 17:600-604 (1957)). In more recently conducted in vitro studies, 6-AN inhibition of tumor cell growth was accompanied by a depletion of purine and pyrimidine nucleotides, and NAD, as well as by a reduction in the ATP to ADP ratio (Hunting et al., Biochem. Pharmacol. 34:3999-4003 (1985)).

The triple combination is less toxic when the interval between courses of PALA + MMPR + 6-AN treatment

is extended from 7 days to 10 or 11 days, and this change in the schedule of administration reduces toxicity sufficiently to permit the safe addition of other drugs (e.g., an apoptosis inducing agent such as FUra) every 10 or 11 days together with the three drug combination.

The PALA-MMPR-6-AN-induced reduction of ATP levels has been demonstrated by both HPLC and the non-invasive technique of NMR spectroscopy (Fig. 1). Although the degree of suppression of ATP was significant at early time points after the triple chemotherapy, by 72 hours the ATP levels had returned to normal. In some normal cells (e.g., hepatocytes), maintenance of severely depressed levels of ATP (20% of control values) for as long as 36-48 hours does not necessarily compromise viability as long as the adenine nucleotide concentrations then return to control values (Farber, Fed. Proc. 32:1534-1539 (1973)). Sustained or permanent loss of ATP is incompatible with cell survival (Schraufstatter et al., J. Clin. Invest. 77:1312-1320 (1986)). However, a temporary (48 hour) depletion of ATP is never the sole determinant compromising cell viability. Any significant drop in ATP concentration has many metabolic consequences, and other induced perturbations to cellular biochemistry (e.g., those induced by PALA and 6-AN) act in concert with ATP loss to cause cell death (Hyslop et al., J. Biol. chem. 263:1665-1675 (1988)). For example, the pentose phosphate shunt provides reducing equivalents in the form of NADPH for certain anabolic reactions and also for the maintenance of reduced glutathione, GSH. GSH is a major cellular reductant (Meister et al., Pharmac. Ther. 49:125-132 (1991), Morrow et al., Cancer Cells 2:15-22 (1990), Doroshow et al., Pharm. Ther. 47:359-370 (1990), Keizer et al., Pharm. Ther. 47:219-231 (1990)), and in the process of detoxification of radical species becomes oxidized glutathione, GSSG. NADPH is required for the conversion of GSSG back to GSH by the enzyme, glutathione reductase. Thus, 6-AN's inhibition of the pentose

phosphate shunt can lead to a lowering of NADPH levels which in turn can prevent adequate GSH resynthesis from GSSH. And, since ATP is required for the initial synthesis of GSH from its constituent amino acids (Meister et al, Pharmac. Ther. 49:125-132, (1991)), the ATP depletion induced by the triple combination can limit supplies of GSH. Thus, the two effects of ATP and NADPH depletion complement each other in this regard, and when the level of GSH falls substantially after chemical injury, cell death usually ensues (Boobis et al., TIPS 10:275-280 (1989)).

The term "biochemical modulation" refers to the pharmacologic manipulation of metabolic pathways by an agent (the modulating agent) to produce the selective enhancement of the antitumor effect of a second agent (the "effector" agent) (Martin, Biochemical modulation -- Perspectives and Objectives. In: New Avenues in Developmental Cancer Chemotherapy. Ed. by Kenneth R. Harrap, London, England (1987)). In this context, the triple combination, PALA + MMPR + 6-AN, may be viewed as biochemical modulation employed to establish in tumor cells a wide array of biochemical changes -- i.e., a primary diminution of ATP lowering of all of the nucleoside triphosphates and NAD, inhibition of macrocmolecular synthesis, and supression of the flux through the pentose phosphate shunt and glycolytic pathways -- thereby establishing a level of disrupted metabolism in cancer cells that complements, and is complemented by the cascade of similar biochemical derangements induced by the apoptosis effects of DNA-damaging anticancer agents (e.g., FUra, cisplatinum, BCNU) (Barry et al., Biochem. Pharmacol. 40:2353-2362 (1990), Berger et al., Anti-Cancer Drug Design, 2:203-210 (1987)). This modulation and complementation results in enhanced cancer cell deaths which reflect in improved tumor regression rates.

Intracellular ATP has been neglected as a primary target for cancer chemotherapy because ATP has long been considered too important a source of energy for all cells to warrant the expectation that primarily directed anti-ATP chemotherapy could be selective for cancer cells and safe for host tissues. However, the subject application documents striking therapeutic activity, a safe therapeutic index, and an associated depletion of ATP levels in the in-vivo treated tumors.

The relative selectivity of the triple therapy for the tumor over host tissues is explained by the finding that many of the enzymes affected by the antimetabolites are in lower concentration in neoplastic than in normal tissues (e.g., NAD-dependent enzymes; Dietrich et al., Cancer Res. 18:1272-1280 (1958), Martin et al., Cancer Res. 17:600-604 (1957), Glock et al., Biochem. J. 65:413-416 (1957), Jedkien et al., J. Biol. Chem. 213:271-280 (1955) and Morton, Nature 18:540-542 (1958)). It is possible to inhibit more selectively enzymes present in cancer tissue in small amounts, while producing much less inactivation of the same enzyme in normal tissues containing larger amounts (Ackerman et al., Proc. Soc. Biol. Med. 72:1-9 (1949)).

The therapeutic activity of a widely diverse group of anticancer agents has been markedly enhanced by this biochemical modulation approach using PALA + MMPR + 6-AN in the CD8F1 murine breast tumor model. The addition of 5-fluorouracil, adriamycin, taxol, radiotherapy, mitomycin C, cis-platinum, cytoxan, phenylalanine mustard, and etoposide with the PALA-MMPR-6-AN triple combination have demonstrated greater anticancer activity that that observed with the individual drugs alone at MTD, or with the PALA-MMPR-6-AN combination alone.

Cellular Energy Depletion Compounds Plus FUra

In one embodiment of the invention cellular energy depletion compounds are administered with 5-fluoroaracil as the apoptosis inducing agent.

In an advantageous embodiment, the subject invention relates to a highly active chemotherapeutic drug combination comprising:

- N-(phosphonacetyl)-L-aspartate (PALA),
- 6-methylmercaptopurine riboside (MMPR),
- 6-aminonicotinamide (6-AN), and
- 5-fluorouracil (FUra).

A quadruple drug combination of PALA + MMPR + 6-AN + FUra, administered in a 10-11 day schedule, produces an impressive partial tumor regression rate.

The underlying molecular mechanism of fluoropyrimidine-induced "thymineless death" has been shown to be due to "programmed cell death" (apoptosis) activated by DNA strand breakage (Kyprianou et al., Biochem. Biophys. Res. Communications 165:73-81, (1989)). Measurements of biochemical changes in CD8F1 breast tumors after in vivo administration of the PALA-MMPR-6-AN drug combination reveal severe ATP loss, inhibition of macromolecular synthesis, inhibition of the pentose phosphate shunt, NAD depletion, reduction of ribonucleoside triphosphates and inhibition of protein synthesis, a pattern of findings which overlap with those reported in "thymineless death" and apoptosis.

In the subject invention, the addition of an apoptosis-inducing anticancer agent such as FUra (Kyprianou et al., Biochem. Biophys. Res. Communications 165:73-81, (1989), Barry et al., Biochem. Pharmacol.

40:2353-2362 (1990)), to the triple combination results in complementary therapeutic activity.

PALA-MMPR-6-AN induces elevation of PRPP levels and reduction of UTP pools. The triple drug combination also increases the therapeutic activity of FUra by increasing the anabolism of FUra to its nucleotides, as well as by favoring the competition of the analog over the natural pyrimidine intermediates whose levels have been reduced by PALA.

The elevation of PRPP levels and the lowering of UTP levels by the triple combination were expected to facilitate the conversion of FUra into its active nucleotides for effective blockade of key FUra-sensitive enzymes, and this was indeed accomplished (Table 5). Thus, the addition of FUra, which at 75 mg/kg as a single agent causes few (<5%) regressions and only inhibits the growth of spontaneous CD8F1 breast tumors, markedly increased the tumor regression rate of the triple combination from 38% to 67% (Table 2).

Three of these drugs (PALA, MMPR, and FUra) currently are used clinically as components of various drug combinations. A combination of PALA plus FUra has proven to be significantly more active than FUra alone in the clinical treatment of colorectal cancer (Ardalan et al., J. Clin. Oncol. 6:1053-1058 (1988), O'Dwyer et al., J. Clin. Oncol. 8:1497-1503 (1990)). MMPR can result in the elevation of PRPP levels in human tumors such as colon, ovary and breast (Peters et al., Cancer Chemother. Pharmacol. 13:136-138 (1984), O'Dwyer et al., J. Natl. Cancer Inst. 83:1235-1240 (1991), Wiemann et al., Med. Oncol. & Pharmacother. 5(2):113-116 (1988)). The addition of MMPR augments the metabolic activation of FUra in human tumors.

Cellular Energy Depletion Compounds Plus Adriamycin

In this embodiment of the invention, cellular energy depletion compounds are administered with adriamycin as the apoptosis inducing agent.

Another advantageous embodiment of the invention comprises:

- N-(phosphonacetyl)-L-aspartate (PALA),
- 6-methylmercaptopurine riboside (MMPR),
- 6-aminonicotinamide (6-AN), and
- Adriamycin (Adria).

This combination yields significantly enhanced anti-cancer activity over that produced by either Adria alone at maximum tolerated dose (MTD), or by the triple drug combination, against large, spontaneous, autochthonous murine breast tumors. The augmented therapeutic results were obtained with approximately one-half the MTD of Adria as a single agent, and provides the clinical benefit of longer and more effective treatment with increased safety. The combination of an ATP-depleting drug combination administered prior to Adria resulted in a 100% tumor regression rate (12% CR; 88% PR) in the population of treated spontaneous tumors indicates that the energy-depleting combination overcomes resistance mechanisms to adriamycin.

Adriamycin has been shown to induce death by apoptosis in cancer cells in vitro (Marks et al., Biochem. Pharmacology 42:1859-1867 (1990)). The addition of Adria to the triple drug combination significantly enhances anti-cancer activity over that produced by either Adria alone at MTD, or by the triple drug combination, against advanced spontaneous, autochthonous murine breast tumors. The augmented therapeutic results are obtained with

approximately one-half the MTD of Adria as a single agent.

The therapeutic results of the most effective single agent against human breast cancer, Adriamycin, are greatly enhanced by the prior administration of the triple combination. These results are obtained with essentially half the dose of the MTD of Adria as a single agent. Adriamycin cannot be given to breast cancer patients for long periods because its cumulative dosage reaches life-threatening heart damage; therefore, the use of much lower doses of Adria, offer the benefit of longer and more effective treatment with increased safety. Further, the clinical development of Adriamycin resistance is frequently based on energy-dependent mechanisms (e.g., multiple drug resistance (mdr) expression: p-glycoprotein) in the cancer cells (Gerlach et al., Cancer Surveys 5:26-46 (1986) and Versantvoort et al., Cancer Res. 52:17-23 (1992)). Results with the triple drug combination are primarily based on depletion of cancer cell energy levels. This ATP-depleting approach in combination with Adria reverses ATP-dependent mechanisms of resistance to Adria in vivo, as has been demonstrated in vitro (Gerlach et al., Cancer Surveys 5:26-46 (1986) and Dano, Biochim. Biophys. Acta. 323:466 (1973)).

The results obtained with the 4-drug combination in CDBF1 mice bearing spontaneous, autochthonous breast tumors represent a significant therapeutic breakthrough in two respects (see Example 2). Firstly, the therapeutic effect of treatment increased from a tumor regression rate of 66% after the first course of treatment to 93 and 100% after second and third courses of treatment, respectively. In contrast, although the first course of Adria alone at its MTD produced a respectable 43% regression rate, the therapeutic value of this drug diminished with subsequent courses of treatment (i.e., 21% regressions after the second course and 16% after the

third course). This decrease in chemotherapeutic activity on successive courses of treatment has been observed in preclinical and clinical trials with Adria, and with most chemotherapeutic drugs. This phenomenon has usually been ascribed to the selection by the drug of cells that were innately resistant to the specific drug, and preferential growth of this sub-population after the drug-sensitive cells have been killed by exposure to the drug. Subsequent drug exposures are confronted with relatively more resistant cells, and the resulting therapeutic observation is drug resistance. As shown in Example 2, the appearance of increased resistance to Adria alone was observable as early as the second course of treatment (on day 10). However, no operational resistance to the PALA - MMPR - 6-AN combination could be detected throughout the 3 courses of treatment regimen and 28 day observation period (Fig. 2). And, importantly, the administration of PALA - MMPR - 6-AN in conjunction with Adria prevented the manifestation of resistance to Adria. In fact, the regression rate observed with the 4-drug treatment increased to 100% after the last course of treatment (Fig. 2).

As mentioned above, the fact that the 4-drug regimen resulted in a 100% tumor regression rate in each of the three successive experiments (a total of 42 spontaneous tumors) was unprecedented in this spontaneous murine breast tumor system. Spontaneous tumors are unlike common transplantable tumor models, which have been repeatedly transplanted for years, and where all of the tumors in a particular experiment, although somewhat heterogeneous on the cellular level, are nevertheless quite similar from one individual host to another. In contrast, spontaneous tumors in these mice, like in humans, differ in drug sensitivity from one individual to another. For example, although on average approximately 20% of the population of spontaneous CD8F1 breast tumors respond with partial tumor regression on exposure to optimal treatment with FUra

alone (Stolfi et al., J. Natl. Cancer Inst. 80:52-55 (1988)), 80% of this population of tumors does not respond to this extent. In fact, if one measures individual tumors during and after treatment with e.g. FUra, one sees an array of responses in individual tumors varying from partial regression to stasis to progression. However, despite this heterogeneity in drug susceptibility among individual tumors, the 4-drug treatment produced regression in all of the tumors in the population. Although this treatment does not overcome heterogeneity in susceptibility completely, as evidenced by the failure to achieve 100% cure of these tumors, this is a new level of therapeutic activity. Further, the spontaneous, autochthonus CD8F1 breast tumor model has demonstrated a remarkable 100% therapeutic correlation with human breast cancer in terms of both positive and negative sensitivity to individual chemotherapeutic drugs using tumor regression as the criterion for evaluation (Stolfi et al., J. Natl. Cancer Inst. 80:52-55 (1988).

Cellular Energy Depletion Compounds Plus Taxol

In this embodiment of the invention cellular energy depletion compounds are administered with taxol as the apoptosis inducing agent.

An advantageous embodiment of the invention comprises:

- N-(phosphonacetyl)-L-aspartate (PALA),
- 6-methylmercaptopurine riboside (MMPR),
- 6-aminonicotinamide (6-AN), and
- taxol.

The above combination yields significantly enhanced anti-cancer activity over that produced by either taxol alone at MTD, or by the triple drug combination, against advanced, first passage spontaneous murine breast tumors. The augmented therapeutic results are obtained with

approximately one-third the MTD of taxol as a single agent. The latter is an important consideration with taxol because of the severe supply problem. Further, taxol resistance is frequently based on energy-dependent mechanisms, and therefore the combination of an ATP-depleting drug combination administered in conjunction with taxol results in the inhibition of energy-dependent resistance mechanisms to taxol.

The new antimicrotubule agent, taxol, an antimitotic agent and the first compound with a taxane ring has demonstrated significant antineoplastic activity in patients with refractory ovarian cancer, refractory breast cancer, non-small cell lung cancer, and other cancers (Rowinsky et al., Pharmac. Ther. 52:35-84 (1991)). Its antitumor activity, novel mechanism of action, and unique structure have generated excitement. Taxol is a plant product (obtained from the bark of the Pacific Yew tree, Taxol brevifolia), and because it is obtained from a limited resource there is a supply problem critical to its widespread clinical use.

Unlike Adria, taxol is not considered a DNA-damaging agent. Taxol is an antimitotic agent that binds preferentially to microtubules in the absence of the cofactors tubulin and GTP, a mechanism unlike that of other antimicrotuble agents in cancer chemotherapeutics (e.g., vincristine, and colchicine). It blocks cells in the mitotic phase of the cell cycle so that these cells are unable to replicate normally, and cell death ensues (Rowinsky et al., Pharmac. Ther. 52:35-84 (1991)). Importantly, however, the taxol-induced disruption of the microtubular network of cancer cells induces death of cancer cells by apoptosis (Martin et al., Cell Tissue Kinet. 23:545-559 (1991), and Lennon et al., Cell Prolif. 24:203-214 (1991)), as Adria does (Marks et al., Biochem. Pharmacol. 42:1859-1867 (1990)).

Another similarity between taxol and Adria is that cells resistant to both agents usually display the multi-drug resistant (MDR) phenotype, p-glycoprotein, an energy-dependent drug efflux pump that maintains each of these agents below an intracellular cytotoxic level (Gerlach et al., Cancer Surveys 5:26-46 (1986), and Endicott et al., Annu. Rev. Biochem. 58:137-171 (1989)). These MDR cells are cross-resistant to both Adria and Taxol (Rowinsky et al., Pharmac. Ther. 52:35-84 (1991)) and Gerlach et al., Cancer Surveys 5:26-46 (1986)), and, since inhibitors of energy production (e.g., azide) when added to such resistant cells in vitro increase the net accumulation of drug in MDR-cells (Gerlach et al., Cancer Surveys 5:26-46 (1986) and Dano, Biochim. Biophys. Acta. 323:466-483 (1973)), the ATP-depleting effects of the PALA-MMPR-6-AN triple combination reverses Adria-and taxol-resistant cells to chemosensitivity.

Still another similarity is that both Adria, and taxol (Holmes et al., Proc. Am. Soc. Clin. Oncol. 10:113 (1991) and Holmes et al., J. Natl. Cancer Inst. 83:1797-1807 (1991)), are effective against human breast cancer. Since the CD8F1 murine breast tumor model has demonstrated a remarkable 100% therapeutic correlation with human breast cancer in terms of both positive and negative sensitivity to individual chemotherapeutic drugs using tumor regression as the criterion for evaluation (Stolfi et al., J. Natl. Cancer Inst. 80:52-55 (1988)), it was deemed likely that taxol, like Adria, also is effective as a single agent against the CD8F1 murine breast tumor. Taxol is similarly enhanced in therapeutic activity by the prior administration of the PALA-MMPR-6-AN triple combination. A markedly lower dose of taxol is necessary in the quadruple combination. Although taxol had been previously reported to be ineffective against the CD8F1 murine mammary carcinoma (Rowinsky et al., Pharmac. Ther. 52:35-84 (1991)), it was considered that the high degree of chemotherapeutic correlation that had been

observed between this model and the human disease warranted re-assessment in the same CD8F1 system.

Example 3 shows that taxol alone is active against the CD8F1 tumor, that the administration of the PALA-MMPR-6-AN triple drug combination prior to taxol significantly enhanced antitumor activity over that produced by either taxol alone at MTD, or the triple drug combination alone, and that only one-third the dose of taxol (in combination) is required for this greater antitumor activity.

Importantly, the antitumor effects of taxol were significantly enhanced by the prior administration of the triple combination, PALA + MMPR + 6-AN, and these therapeutic results were obtained with one-third the MTD of taxol as a single agent (Table 2 and 3). The potential benefit, in addition to enhanced clinical responses with taxol in human breast cancer, is that the ability to employ lower effective doses of taxol should alleviate the taxol supply problem. An additional benefit of this particular drug combination is that, since acquired taxol resistance involves the mdr phenotype with p-glycoprotein as an energy-dependent drug effux pump (Rowinsky et al., Pharmac. Ther. 52:35-84 (1991)), this ATP-depleting approach in combination with taxol overcomes ATP-dependent mechanisms of resistance to taxol.

Cellular Energy Depletion Compounds Plus Radiation

In this embodiment of the invention, cellular energy depletion compounds are administered with radiation as the apoptosis inducing agent.

The PALA-MMPR-6-AN combination sensitizes tumors to ionizing radiation therapy (see Example IV).

Therapeutic Uses of the Compositions of the Invention

The drug combinations of the subject invention are useful in treating a wide variety of neoplastic diseases. For a particular type of cancer, the apoptosis inducing agent of the combination of the invention (which comprises the cellular energy depletion compounds plus an apoptosis inducing agent), is selected on the basis of demonstrated antitumor activity when it is used without the cellular energy depleting combination. The cellular energy depletion compounds (e.g. PALA-MMPR-6-AN) are primarily intended to sensitize tumors to apoptosis inducing agents to which the tumors are already susceptible to some degree.

For example, fluorouracil (and therefore, the combination of the invention comprising fluorouracil and the cellular energy depletion compounds, e.g. PALA-MMPR-6-AN) is useful for treating tumors of the colon, stomach, breast, head-and-neck, and pancreas. Taxol (and therefore the combination of taxol with the cellular energy depletion compounds) is useful for treating cancers of the ovary and breast. Adriamycin (and therefore the combination of adriamycin with the energy depletion compounds of the subject invention) is useful in a wide variety of tumors: acute leukemias, malignant lymphomas, cancers of the ovary, breast, lung, bladder, thyroid, endometrium, testes, prostate, cervix, head-and-neck, and in osteogenic and soft tissue sarcomas (The Pharmacological Basis of Therapeutics, Seventh Edition (1985), ed.A.G. Gilman, L.S. Goodman, T.W. Rall, and F. Murad, Macmillan Publishing Company, New York, Ny, pp. 1283-1285). Ionizing radiation (and therefore the combination of ionizing radiation with the cellular energy depletion compounds of the invention) is useful for treating a variety of tumor types, including, lymphomas, and cancers of the breast, pelvis, and lung.

Furthermore, since the energy depleting combinations of the subject invention render tumors more sensitive to each apoptosis inducing agent, the range of tumors susceptible to treatment with a given apoptosis inducing agent in combination with the cellular energy depleting combinations of the invention is broader than the range of tumor types treatable with the apoptosis inducing agent alone.

A combination of two or more apoptosis inducing agents is optionally administered in conjunction with an energy depleting composition of the invention.

The cellular energy depleting combination of the invention is also useful for overcoming multiple drug resistance in patients. "Multiple drug resistance" (MDR) is a condition in which tumor cells become insensitive to a variety of cytotoxic antineoplatic agents. MDR is typically due to the presence of an energy-requiring pump system which removes cytotoxic agents including adriamycin, taxol, and vinca alkaloids from cells (or critical regions of cells, such as the nucleus) (Gervasoni et al., Cancer Research, 51:4955-4963 (1991)). In Example 2, in mice treated with adriamycin alone, the response to adriamycin decreases over time during the three courses of drug treatment, indicating that the tumors are developing resistance to the drug. In contrast, in mice treated with adriamycin plus an energy depleting combination of the invention, the response rate actually improves over time during the three courses of treatment, indicating that the tumors are not developing resistance to adriamycin, and in fact, an unprecedented 100% partial regression rate was observed in these animals (Figure 2).

Administration and Formulation of the Compositions of the Invention

The compounds of the invention are administered in

therapeutically effective amounts. The term "therapeutically effective amount" as used herein refers to that amount which provides therapeutic effects for a given condition and administration regimen. The compounds and compositions of the invention are administered orally, by parenteral injection, intravenously, or topically, depending on the condition being treated.

The timing and sequence of administration of the compounds and compositions of the invention affect the efficacy of the treatment. Typically, an inhibitor of pyrimidine biosynthesis (e.g. PALA) is administered 10 to 24 hours prior to administration of an inhibitor of purine biosynthesis and a nicotinamide antagonist. An inhibitor of purine nucleotide biosynthesis (e.g. MMPR) and a nicotinamide antagonist (e.g. 6-AN) are typically administered at approximately the same time. The apoptosis inducing agent(s) is administered after the cellular energy depleting combination, typically about 2 to 3 hours afterwards (although the timing may be modified for particular drugs according to observed clinical benefit).

The doses of the particular agents are determined according to clinical response, alterations in biochemical indices of efficacy, and observed signs of toxicity.

PALA is typically administered in a dose of 250 mg/square meter; this dose has been found to be suitable in clinical studies in which PALA was administered as a modulator of fluorouracil. A typical single dose of 6-AN is 10 to 50 mg/square meter. The optimum dose range for 6-AN is determined by taking tissue biopsies before and after administration of increasing doses of 6-AN and determining activity of enzymes requiring pyridine nucleotides, e.g phosphogluconate dehydrogenase. The

courses of treatment. A typical single dose of MMPR is 200 to 400 mg/square meter; a dose of 225 mg/kg has been safely administered to humans in combination with PALA.

The compounds and compositions of the invention are formulated in pharmaceutically acceptable carriers. The doses of the components of the energy-depleting combinations are chosen with the intention of providing significant sensitization of tumors to apoptosis-inducing agents. If undue toxicity due to one or more of the energy-depleting compounds is encountered, subsequent doses or the interval between courses of treatment are modified. Alternatively, appropriate antidotes are administered: Niacin or niacinamide ameliorate toxicity due to 6-AN (or other nicotinamide antagonists; uridine, prodrugs of uridine, or other pyrimidine nucleotide precursors reverse the biochemical deficits produced by PALA or other inhibitors of pyrimidine nucleotide biosynthesis; and cellular purine nucleotide pools affected by MMPR (or other inhibitors of purine nucleotide biosynthesis) are replenished by administering appropriate purine nucleotide precursors, e.g., inosine, AICAR, adenosine, or hypoxanthine, with or without an inhibitor of purine degradation, such as allopurinol.

Doses of apoptosis-inducing agents are chosen to optimize the therapeutic index. Since the energy-depleting combination sensitizes tumors to apoptosis-inducing agents, doses of apoptosis-inducing agents are typically less than or equal to the doses that would be administered in the absence of the energy-depleting composition. As is demonstrated in Examples II and III, pretreatment with PALA-MMPR-6-AN permits substantial reductions in the doses of adriamycin and taxol required to produce optimum benefit. This is very important, since adriamycin produces cumulative cardiotoxicity, limiting the total amount that a patient can safely receive. Supplies of taxol are currently

limited (since it is obtained from the bark of the Pacific Yew, a relatively uncommon tree). A substantial reduction in the dose of taxol needed to produce an optimum clinical effect (as is demonstrated in Example III), permits more patients to be effectively treated with taxol (or other scarce but effective apoptosis-inducing agents).

A course of treatment (PALA-MMPR-6-AN followed by an apoptosis-inducing agent) is repeated about every 10 days. Patients typically receive three or more consecutive courses of treatment. The precise number of courses of treatment and the interval between is adjusted by the skilled person according to clinical determination of efficacy and toxicity.

For parenteral administration by injection or intravenous infusion, the compounds and compositions of the invention are dissolved or suspended in aqueous medium such as sterile physiological saline. In the case of poorly-soluble compounds (e.g. taxol), solubilizing agents like ethanol, propylene glycol, or polyoxyethylated castor oil are used.

* * *

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

THE EXAMPLES

Example I: PALA, MMPR, 6-AN and FUra

Murine Breast Tumor System: Spontaneous, Autochthonous Mammary Carcinoma -- CD8F1 hybrid mice bearing single spontaneous, autochthonous breast tumors arising during the preceding week were selected from a colony which has been described previously (Stolfi et al.,

Cancer Chemother. Rep. 55:239-251 (1971), Martin et al., Cancer Chemother. Rep., Part 2, 5:89-109 (1975)). All tumors were measured with calipers, and the mice were distributed among experimental groups so that mice carrying tumors of approximately equal weight were represented in each treatment group. Individual tumors ranged in size from 100 to 500 mg, and the average tumor weight in all groups was 260 mg at the beginning of treatment.

Murine Breast Tumor System: First Passage CD8F1 Mammary Carcinoma -- For each experiment, the spontaneously arising CD8F1 breast tumors were transplanted into syngeneic three-month old mice. As in all spontaneous tumors, whether human or murine, each individual cancer has a heterogeneous neoplastic cell population. The first generation transplants of CD8F1 breast tumors are obtained from a tumor cell brei made by pooling 3-4 spontaneously-arising tumors. Thus, the individual transplants in each experiment develop from a single brei that, although common to all the mice in that experiment, has a neoplastic cell composition that is likely slightly different from that in another experiment. Therefore, quantitative measurements of any individual parameter (e.g., TSase activity, or average tumor size) may be somewhat different from experiment to experiment, but the findings will be quantitatively relevant within individual experiments, as will similar trends among experiments. The CD8F1 first generation breast tumor is included in the murine tumor testing panel of the National Cancer Drug Screening Program (Goldin et al., Eur. J. Cancer 17:129-142 (1981)).

In approximately three to four weeks, when transplanted tumors were measurable, the tumor-bearing mice were distributed among experimental groups so that mice carrying tumors of approximately equal weight were

represented in each treatment group. The average tumor weight was close to 125 mg at the beginning of treatment.

Tumor Measurements -- Two axes of the tumor (the longest axis, L, and the shortest axis, W) were measured with the aid of a Vernier caliper. Tumor weight was estimated according to the formula: tumor weight (mg) = L (mm) x $(W(MM)^2)/2$.

Chemotherapeutic Agents -- MMPR, 6-AN and FUra were obtained from Sigma Chemical Co., St. Louis, MO. Each of these agents was dissolved in 0.85% NaCl solution immediately before use. PALA was obtained from the Department of Health, Education, and Welfare, USPHS of the National Cancer Institute, Bethesda, MD. PALA was dissolved in 0.85% NaCl solution, and the pH was adjusted to 7.2 to 7.5 with 1N NaOH before adjustment to final volume. All agents were administered i.p. so that the desired dose was contained in 0.1 ml/10 g of mouse body weight.

These drugs were administered in a timed sequence, with PALA administered 17 hours before the simultaneous administration of MMPR + 6-AN, and with 5-FUra administered 2½ hours after MMPR + 6-AN. Throughout this application, the timing of biochemical measurements is given in relation to the injection of the last chemotherapy (i.e., MMPR + 6-AN) in the chemotherapeutic sequence.

Determination of Chemotherapy-Induced Tumor Regression Rate -- The initial size of each tumor in each treatment group was recorded prior to the initiation of treatment. Tumor size was recorded weekly during treatment and again at 7-9 days after the last course of treatment. For each experiment a single observer made all measurements in order to avoid variation in caliper measurements from individual to individual. By

convention, partial tumor regression is defined as a reduction in tumor volume of 50% or greater compared to the tumor volume at the time of initiation of treatment. The partial regression rate obtained from a particular treatment is expressed as a percentage; i.e., number of partial regressions per group/total number of animals per group x 100.

Statistical Evaluation -- Differences in the number of partial tumor regressions between treatment groups were compared for statistical significance by chi-square analysis. Students t test was used for evaluation of measured biochemical differences between treatment groups. Differences between groups with $p \leq 0.05$ were considered to be significant.

Incorporation of Precursors into RNA and DNA -- Radiolabeled precursors, ^{32}P and (3H)-L-Leucine were administered i.p.; the labeling period was 2 hrs: At the end of the labeling period, animals were sacrificed by cervical dislocation. Tumor tissues were homogenzied in TNE buffer containing 1% Triton-X 100 (TNE: 0.01 M Tris-HCl, pH 7.6; 0.15M NaCl; 0.001 M EDTA). The homogenate was briefly sonicated, and then digested with Pronase for 60 min. at 37°C (0.2 mg/ml for 2 hr. at 37°C) and finally, this material was extracted with chloroform/isoamyl alcohol (24:1 vol/vol). Samples of extracted material were precipitated with trichloroacetic acid to determine total radioactivity. Other samples are first treated with alkali (0.4M NaOH, for 90 min at 37°C) to determine alkali-stable, trichloroacetic acid-precipitable radioactivity. The difference between the total and alkali-stable radioactivity was assumed to represent radioactivity in RNA. (5-^{3}H)Fluorouracil (20 Ci/mmol), (5-^{3}H)$_3$ thymidine and (5-^{3}H)deoxyuridine monophosphate (20 Ci/mmol) were purchased from Moravek.

Substrate Accumulations -- Cellular levels of 6-phosphogluconate, glucose-6-phosphate and fructose-6-phosphate were measured on perchloric acid extracts by published methods (6-phosphogluconate (Haid, Methods of Enzymatic Analysis (Bergmeyer, H.U., ed). New York: Academic Press pp. 1248-1250 (1974)); glucose-6-phosphate and fructose-6-phosphate (Lang et al., Methods of enzymatic analysis, Vol. 2 (ed. H.U. Bergmeyer)).

^{14}C-Orotic Acid Assay for PRPP(5-phosphoribosyl pyrophosphate) -- The assay is based on the conversion of ^{14}C-orotic acid to uridine monophosphate with release of $^{14}CO_2$ by orotidine-5-phosphate- pyrophosphorylase + orotidine-5-phosphate decarboxylase (Houghton et al., Mol. Pharmacol. 22:771-778 (1982)). An aliquot of the homogenate was assayed for protein content by the method of Lowry et al., J. Biol. Chem. 193:265-275 (1951).

Processing of Samples for NTP Content -- Frozen tumor specimens were homogenized in ice-cold 1.2 N perchloric acid. The acid-insoluble fraction was removed by centrifugation (7000 rpm for 15 min). The acid-soluble fraction as neutralized by extraction with a mixture of freon and tri-N-octylamine (2:1). The extract was then filtered through a 0.22 u Millipore membrane filter prior to HPLC analysis. NTP contents in tumor were normalized to the protein content of the acid-insoluble fraction.

Measurements of NTP Levels in Tumor -- HPLC analysis in tumor was performed on a Waters 840 HPLC system with a WISP automatic sampler. NTP levels were analyzed by ion-exchange gradient chromatography using a Waters SAX column starting with 3 mM $NH_4H_2PO_4$, pH 3.5, proceeding in two steps to 70% 0.5 M $NH_4H_2PO_4$, pH 5.0, plus 30% starting buffer. The run time for each 100 ul of extracted sample was 60 min. Tumor NTP levels are expressed as micrograms nucleotide triphosphate per milligram of protein.

31P NMR (Nuclear Magnetic Resonance) Spectra -- 31P NMR spectra were obtained using techniques described previously (Koutcher et al., Cancer Res. 50:7252-7256 (1990)). Briefly, spectra were obtained on a General Electric NT-300 wide bore spectrometer operating at 121.5 MHz. Experimental parameters included a spectral width of +/- 12,000 Hz, 60° tip angle, recycle delay of 2 seconds, 512-1024 averaged free induction decays (FID's), and 1024 data points. The spectra are partially saturated using these experimental conditions. Four turn solenoid coils with a Faraday shield (Ng et al., J. Magn. Reson. 49:526 (1982)), positioned between the body of the mouse and the coil were used to detect the NMR signal. Control experiments verified that no signal was obtained from non-tumor bearing animals mounted in an identical manner. Spectra were analyzed using 25 Hz exponential multiplication followed by Fourier transformation. Spectral peak areas were estimated by fitting the spectra to a series of Lorentzian peaks, using a program (GEMCAP) available on the spectrometer, after fitting the baseline to a third order polynomial (using standard General Electric software). Since other peaks overlap the alpha- and gamma-nucleotide triphosphate (NTP) peaks, the beta-NTP was used for calculating NTP peak area ratios.

Thymidine kinase and Thymidylate synthase assays -- Tissues were homogenzied (Potter-Elvehjem homogenizer) as a 20% (wt/vol) solution in Tris-Cl (100 mM, pH 7.6), 2-mercaptoethanol (20 mM) and sodium fluoride (100 mM). Homogenates were centrifuged at 4 (100,000 x g, 60 min. or 10,000 x g, 30 min) and the supernatant fractions retained on ice. Enzyme assays were performed either on individual samples or on pooled tissues from three animals. Tumors were between 300-500 mg. Thymidine kinase was measured immediately after cytosol preparation by means of a DE81-filter-binding assay (Ives et al., Anal. Biochem. 28:192-205 (1969)). The assay mixture contained Tris-Cl (100 mM, pH 7.6), ATP (5 mM), $MgCl_2$ and (5-C^3H_3)thymidine

(25 uM, 1.0 Ci/mmol) and cytosolic protein. Thymidylate synthase activity was measured by the release of tritium from (5-^{3}H)dUMP (10 uM, 1.0 Ci/mmol), CH_2H_4PteGlu (100 uM) and cytosolic fraction (25 uL) (Roberts, Biochemistry 5:3546-3548 (1966)). Reactions were terminated by the addition of perchloric acid (10 uL, 0.7M). Protein was determined by the method of Lowry et al., J. Biol. chem. 193:265-275 (1951).

Incorporation of FUra into RNA -- Incorporation of FUra into RNA was determined by isolating tumor RNA by the acid-guanidine isothiocyanate procedure (Chomczynski et al., Anal. Biochem. 162:156-159 (1987)), after treatment with (5-^{3}H)FUra (2.0 mCi/mmol). Tissues were harvested 4-5 hr after treatment, immediately frozen in liquid nitrogen and stored at -70. Tissues were homogenized (80 mg/ml) in the denaturing solution (citrate-sarcosyl laurate-2 mercaptoethanol) and extracted with one volume of phenol:chloroform:isoamyl alcohol (50:40:2). After extraction the RNA was precipitated with alcohol, washed, redissolved and quantitated by uv, and then precipitated with perchloric acid. This final precipitate was collected on GF-C filters and the radioactivity was determined.

Chemotherapeutic Effects on Breast Tumor Transplants

In a series of 5 similar experiments, a group (Group 1) of CD8F1 mice bearing advanced, first passage spontenaous CD8F1 breast tumor transplants were treated with the triple combination of PALA + MMPR + 6-AN. PALA (100 mg/kg) was administered 17 hours before MMPR (150 mg/kg) plus 6-AN (10 mg/kg) (Table 1). A second group (Group 2) received the same treatment with PALA + MMPR + 6-AN, followed 2½ hours later by FUra (75 mg/kg). Treatment was repeated at 10 or 11 day intervals for a total of 3 courses, and observations were recorded 7 days after the last course of treatment.

Table 1

Therapeutic Comparison of PALA + MMPR + 6-AN Chemotherapy With and Without the Addition of 5-Fluorouracil (FUra) in CD8F1 Mice Bearing First Passage Syngeneic Breast Tumors[a]

Treatment[b]	Percent Body Weight Change	Dead/Total	Partial Regressions
1. $PALA_{100}$-17 hr->$MMPR_{150}$ + $6\text{-}AN_{10}$	-16	1/49 (2%)	8 (17%)
2. $PALA_{100}$-17 hr->$MMPR_{150}$ + $6\text{-}AN_{10}$-2½ hr->$FUra_{75}$	-22	0/50 (0%)	37 (74%)
3. $FUra_{75}$			0/88

[a] Pooled data: Experiments 2055F, 2076M; 2091F, 2102F, 2113F. First passage CD8F1 breast tumors averaging 125 mgs at the time of initiation of treatment.

[b] Three courses of the indicated treatment were administered with a 10 or 11 day interval between courses. Subscripts refer to doses in mg/kg. Observations were recorded 7 days after the third course of treatment.

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Partial tumor regressions were observed in 8 of the surviving 48 mice (17%), treated with PALA + MMPR + 6-AN (Group 1). The range of regression rate in individual experiments varied from 0 to 30%. The addition of FUra to the same regimen of PALA + MMPR + 6-AN (Group 2) produced a significant and meaningful increase in therapeutic activity. Thirty-seven of the 50 treated mice (i.e., 74%) experienced partial tumor regressions (with a range in the individual experiments of 60-90%). This level of therapeutic activity was found to be significantly better than that achieved with the three-drug combination (without FUra, Group 1, $p < 0.001$), and most importantly, this increase in antitumor activity was achieved without mortality. It should be noted that FUra alone at 75 mg/kg did not produce any tumor regressions after 3 courses of treatment in nine separate experiments (0/88 mice, not shown).

In 3 separate experiments the therapeutic activity of the PALA + MMPR + 6-AN + FUra combination was compared with that of the combination of PALA + MMPR + FUra (i.e., without 6-AN) at the same doses and administration schedule. One week after three courses of treatment the four-drug combination yielded a 71% tumor regression rate (20 tumor regressions in 28 surviving mice) whereas the three-drug combination without 6-AN yielded a regression rate of only 41% (12 tumor regressions in 29 surviving mice). The difference in regression rate between these two treatments was found to be statistically significant, $p < 0.05$, indicating that the low-dose 6-AN was contributing to the therapeutic activity in the four-drug combination.

Chemotherapeutic Effects on Spontaneous, Autochthonous Breast Tumors

The PALA + MMPR + 6-AN regimen, with or without FUra, was administered at 10-11 day intervals in a series of six

experiments to large numbers (a total of 66 or 67 mice in each treatment arm) of CD8F1 mice bearing advanced, spontaneous, autochthonous breast tumors averaging 260 mgs. Results were observed at 6 weeks after the initiation of treatment (i.e., 9 days after the fourth course of treatment) (Table 2).

Table 2

Therapeutic Comparison of PALA + MMPR + 6-AN Chemotherapy With and Without the Addition of 5-Fluorouracil (FUra) in Mice Bearing Spontaneous, Autochthonous CD8F1 Breast Tumors[a]

Treatment[b]	Percent Body Weight Change	Dead/Total	Partial Regressions
1. $PALA_{100}$-17hr->$MMPR_{150}$ + $6\text{-}AN_{10}$	-10	3/67 (4%)	24 (38%)
2. $PALA_{100}$-17hr->$MMPR_{150}$ + $6\text{-}AN_{10}$-2½->$FUra_{75}$	-10	5/66 (7%)	41 (67%)
3. $FUra_{75}$			25%

[a] Pooled data: Experiments R536; R537; R538; R539; R540; R541. Spontaneous, autochthonous CD8F1 breast tumors averaging 260 mgs at the time of initiation of treatment.

[b] The indicated treatment was administered at 10-11 day intervals. Subscripts refer to doses in mg/kg. Observations were recorded 6 weeks after initiation of treatment (i.e., approximately 9 days after the fourth course of treatment).

The PALA + MMPR + 6-AN regimen (group 1) produced a partial tumor regression rate of 38% in mice bearing spontaneous, autochthonous breast tumors (24 partial tumor regressions in 64 surviving mice) with an acceptable mortality rate (4%, 3 deaths in 67 treated mice). The addition of FUra to the three-drug regimen (Group 2) resulted in 41 partial regressions in the 61 surviving mice, or 67% (with a range of 50-90% in the individual experiments) without an increase in the mortality rate (only 7%), and with only a 10% body weight loss. As in the previous experiments in mice bearing first passage tumors, the addition of FUra to the PALA, MMPR, 6-AN regimen resulted in a significant increase in tumor regressions ($p < 0.01$) in mice bearing spontaneous, autochthonous breast tumors. Again, it should be noted that FUra alone at 75 mg/kg produced < 5% regressions of spontaneous, autochthonous CD8F1 breast tumors (1 regression in 24 treated tumors), and spontaneous regressions of these tumors has not been observed.

BIOCHEMICAL FINDINGS

Macromolecular Synthesis -- Administration of PALA-MMPR-6-AN resulted in significant inhibition of macromolecular synthesis in first passage CD8F1 breast tumors detectable at the earliest time point examined, 2.5 hours after drug administration, and progressing to 80% inhibition of DNA synthesis, 85% inhibition of RNA synthesis and 70% inhibition of protein synthesis at 48 hours.

Pentose Shunt and Glycolytic Intermediates

6-AN has been reported to inhibit the pentose phosphate pathway by producing a metabolic block on 6-phosphogluconate (6-PG) dehydrogenase (Herken et al., Biochem. Biophys. Res. Comm. 36:93-100 (1969)). The

accumulation of this substrate results in feed-back inhibition of phosphoglucose isomerase and prevents the formation of fructose-6-phosphate from glucose-6-phosphate (G-6-P) (Racker, In: Mechanisms in Bioenergetics. Academic Press, New York-London, p. 207 (1965) , Horecker, B.C. In: Carbohydrate Metabolism and Its Disorders. Vol. I. Academic Press, New York-London (1968)). As is evident in Table 3, administration of 6-AN did, in fact, result in significant elevation of 6-PG (167-fold increase above saline-treated control), and G-6-P (3-fold increase above control) in CD8F1 breast tumors. Similar results were obtained in tumors from mice treated with the 6-AN-containing 3-drug combination (PALA-MMPR-6-AN). The 3-drug combination reduces NAD levels in this tumor (Martin, Metabolism and Action of Anti-cancer drugs. Ed. by Garth Powis and Russell A. Prough (London: Taylor & Francis), pp. 91-140 (1987)).

Table 3

Effects of $PALA_{100}$--17 hrs--> $MMPR_{150}$ + $6\text{-}AN_{10}$ On Substrate Concentrations (umol/g frozen weight) Of Pentose Shunt and Glycolytic Intermediates

Group	Treatment	6-Phospho Gluconate (6-PG)[a]	p value*	Glucose 6-Phosphate (G-6-P)[b]	p value*
1	Saline	0.009 ± 0.001	--	0.036 ± 0.004	--
2	$6\text{-}AN_{10}$	1.5 ± 0.165	0.02	0.116 ± 0.017	0.02
3	$PALA_{100}$--17hr--> + $MMPR_{150}$ + $6AN_{10}$	0.93 ± 0.053	0.01	0.087 ± 0.018	0.05

Exp. JC28; JC37; CD8F1 breast tumors in CD8F1 female mice; subscripts = mg/kg body weight; i.p. injections; 6 hours after last chemotherapy.
*Statistical comparison to Group 1 (saline control)
a Values represent mean ± S.E. of 3 tumors/group
b Values represent mean ± S.E. of 6 tumors/group.

Estimation of Cellular Energy Levels by NMR (See Fig. 1) -- NMR spectra were obtained from first passage CD8F1 breast tumors prior to treatment, and at 2, 10, and 24 hours post treatment with PALA-MMPR-6-AN. Baseline spectra were similar to those obtained in previous studies (Koutcher et al., Magnetic Resonance in Medicine 19:113-123 (1991)). Post-treatment, a decrease in the B-NTP peak relative to inorganic phosphate was noted. The results obtained from 7 tumor-bearing animals treated in this manner are summarized in Fig. 1 which shows that both phosphocreatine/inorganic (PCr/Pi) and NTP (nucleoside triphosphate)/Pi ratios were decreased after treatment with this drug combination, which is indicative of energy depletion. The changes in PCr/Pi and NTP/Pi at 10 hours were found to be statistically significant.

Biochemical Measurement of ATP Levels

Energy depletion in drug-treated tumors also was manifest in decreased ATP pools as shown in Table 4. At 6 and 24 hours after administration of MMPR alone (Group 2), 6-AN alone (Group 3), or the 3-drug combination, PALA-MMPR-6-AN (Group 4), ATP levels in first passage CD8F1 breast tumors were significantly depressed, reaching a level of 32% of control in tumors from mice treated with the 3-drug combination at 24 hours post treatment.

Table 4

Effect of $PALA_{100}$ 17 hr $MMPR_{150}$ + $6\text{-}AN_{10}$ on Tumor ATP Pools[a] in CD8F1 Female Mice (ug/mg protein ± S.E.)

Group	Treatment	ug ATP/mg protein ( )[b] 6 hr	24 hr
1	Saline	7.1 ± 0.32	7.1 ± 0.32
2	$MMPR_{150}$	3.3* (47%) ± 0.32	3.3* (47%) ± 0.96
3	$6\text{-}AN_{10}$	5.5* (77%) ± 0.21	5.3* (75%) ± 0.13
4	PALA 17 hrs $MMPR_{150}$ + $6\text{-}AN_{10}$	3.9* (55%) ± 0.11	2.3* (32%) ± 0.31

[a] Mean ± S.E. of 10 tumors/group (11 separate experiments) at indicated times after last chemotherapy. Subscripts = mg/kg body weight; i.p. injection.

* Significant = p value less than or equal to 0.05;

[b] ( ) = % of saline control (Group 1).

Biochemical Changes Favoring the Activation and Competitive Activity of FUra -- As a consequence of the block of de novo purine synthesis, the administration of MMPR has been shown to result in the accumulation of PRPP in CD8F1 tumor cells, and when MMPR is administered appropriately before FUra, the increased levels of PRPP result in enhanced activation of FUra (Martin et al., Nucleosides and Cancer Treatment (M.H.N. Tattersal and R.M. Fox, eds.), Academic Press, Australia, pp. 339-392 (1981)). Because of the possibility of complex interactions among drugs in combination, it was necessary to verify that the MMPR-containing 3-drug combination also was capable of producing this elevation of PRPP in tumor cells. Accordingly, PRPP levels were measured in untreated first passage CD8F1 tumor, and in CD8F1 tumors at 3 and 24 hours after treatment with PALA-MMPR-6-AN. Measurements from 4 untreated tumors in each of 3 separate experiments yielded an average PRPP level of 288 pmol/mg with a standard error of 19 pM/mg. PRPP levels rose to 490 ± 63 pmol/mg (i.e., 2.2-fold increase, $p < 0.01$) and 833 ± 153 pmol/mg (i.e., 3.7-fold increase, $p < 0.05$) at 3 and 24 hours, respectively, after administration of PALA-MMPR-6-AN.

As a consequence of the inhibition of aspartate transcarbamylase, the administration of PALA alone has been demonstrated to result in depletion of UTP pools in CD8F1 tumors, and when administered appropriately before FUra, this results in an augmentation of the activity of the competitive FUTP analog (Martin et al., Cancer Res. 43:2317-2321 (1983)). Again, UTP levels were measured in first passage CD8F1 breast tumors at 24 hours after administration of PALA-MMPR-6-AN and found statistically significant depression of UTP pools compared to saline-treated control tumors (data not shown).

Therefore, two of the drugs (MMPR and PALA) in the 3-drug combination produce the same biochemical alterations in CD8F1 breast tumor cells when administered in the 3-drug combination as they did when administered as single agents, and these alterations have been demonstrated to result in the augmentation of subsequently administered FUra.

Biochemical Measurements of FUra Activity when Administered Two- and One-half Hours after PALA-MMPR-6-AN

Since the inhibition of RNA synthesis is one of the earliest measurable events after the administration of the triple combination, the effect of the combination upon the incorporation of FUra into RNA is of interest. The amount of tumor (FU)RNA in the group which received PALA + MMPR + 6-AN + FUra was appreciable (355 ± 127 cpm/mg RNA). Thus, although RNA synthesis was significantly inhibited following treatment with PALA-MMPR-6-AN, the incorporation of FUra into residual newly synthesized RNA was not inhibited.

Three groups of 5 CD8F1 mice bearing equi-sized first passage breast tumors were treated with saline, PALA-MMPR-6-AN, or PALA-MMPR-6-AN followed 2.5 hours later with FUra. Measurements of thymidine kinase and thymidylate synthase activity 24 hours after treatment are shown in Table 5.

Table 5

Tumor Thymidine Kinase and Thymidylate Synthase Activities
24 Hours After[+] Treatment

Treatment	% of Control Activity TKase[*]	% of Control Activity TSase[**]
1. Saline	100.00 ± 22.30	100.00 ± 18.31
2. $PALA_{100}$ 17 hr $MMPR_{150}$ + $6\text{-}AN_{10}$	19.63 ± 4.28	53.40 ± 13.61
3. $PALA_{100}$ 17 hr $MMPR_{150}$ + $6\text{-}AN_{10}$ 2.5 hr FU_{75}	6.26 ± 1.61	23.60 ± 3.07

[+]Tumors harvested 24 hours after MMPR + 6-AN treatment; N = 5.
[*]avg. control activity = 46.0 pmol/min/mg protein
[**]avg. control activity = 12.71 pmol/min/mg protein

Thymidylate synthase activity was decreased by nearly 50% after treatment with PALA-MMPR-6-AN, even without the addition of FUra (Group 2, Table 5). This depression of enzyme activity is likely due to the general inhibition of protein synthesis following treatment with the 3-drug combination described above. However, the addition of FUra 2.5 hours following treatment with the 3-drug combination (Group 3, Table 5) resulted in more than 76% inhibition of this enzyme. This increased level of inhibition is believed to be a result of FdUMP inhibition of residual thymidylate synthase activity.

Nord et al., Biochem. Pharmacol. 42:2369-2375 (1991), reported that thymidine kinase activity is lost over 24 hour in CD8F1 tumors from mice treated with the maximum tolerated dose (100 mg/kg) of FUra alone. The triple combination (Group 2, Table 5) produced approximately 80% inhibition of TKase (apparently due to inhibition of RNA synthesis), while the addition of FUra (75 mg/kg) to the triple combination (Group 3, Table 5) provided further inhibition (93.7%) of TKase activity.

Therapeutic activity has been measured in these studies using stringent clinical criteria of tumor regression (i.e., 50% or greater drug-induced decrease in tumor size), rather than the more conventional animal model criteria of tumor growth inhibition. It should also be noted that the spontaneous, autochthonous CD8F1 breast tumor model has demonstrated a remarkable correlation with human breast cancer in terms of both positive and negative sensitivity to individual chemotherapeutic drugs using tumor regression as the criterion for evaluation (Stolfi et al., J. Natl. Cancer Inst. 80:52-55 (1988)).

This example shows an impressive increase in tumor regression rates when FUra was administered in conjunction with PALA, MMPR and 6-AN in the therapy of either advanced first passage, or spontaneous, murine breast tumors, and

shows the results of measurements of biochemical parameters affected by treatment.

Example 2: PALA, MMPR, 6-AN and Adriamycin

Murine Breast Tumor System
Spontaneous, Autochthonous Mammary Carcinoma

CD8F1 hybrid mice bearing single spontaneous, autochthonous breast tumors arising during the preceeding week were selected from a colony which has been described previously (Stolfi et al., Cancer Chemother. Rep. 55:239-251 (1971) and Martin et al., Cancer Chemother. Rep., Part 2, 5:89-109 (1975)), and is included in the murine tumor testing panel of the National Cancer Drug Screening Program (Goldin et al., Eur. J. Cancer 17:129-142 (1981)). All tumors were measured with calipers, and the mice were distributed among experimental groups so that mice carrying tumors of approximately equal weight were represented in each treatment group. Individual tumors ranged in size from 100 to 500 mg, and the average tumor weight in all groups was 304 mg at the beginning of treatment. As in all spontaneous tumors, whether human or murine, each individual cancer has a heterogeneous cell population and therefore, unlike long-transplanted tumor lines, one spontaneous tumor may differ from another of the same histiotype in susceptibility to a given drug treatment.

Tumor Measurement

Two axes of the tumor (the longest axis, L and the shortest axis, W) were measured with the aid of a Vernier caliper. Tumor weight was estimated according to the formula: tumor weight (mg) = L (mm) x $(W(mm)^2)/2$.

Chemotherapeutic Agents

MMPR and 6-AN were obtained from Sigma Chemical Co., St. Louis, MO. Adria was obtained from Adria Laboratories, Columbus, Ohio. Each of these agents was dissolved in 0.85% NaCl solution immediately before use. PALA was obtained from the Department of Health, Education, and Welfare, USPHS of the National Cancer Institute, Bethesda, MD. PALA was dissolved in 0.85% NaCl solution, and the pH was adjusted to 7.2 to 7.5 with 1N NaOH before adjustment to final volume. All agents were administered so that the desired dose was contained in 0.1 ml/10 g of mouse body weight. Adria was administered i.v. and all other agents were administered i.p.

These drugs were administered in a timed sequence, with PALA administered 17 hours before MMPR + 6-AN, and Adria administered 2 1/2 hours after MMPR + 6-AN.

Determination of Chemotherapy-Induced Tumor Regression Rate

The initial location and size of each tumor in each treatment group was recorded prior to the initiation of treatment. Tumor size was recorded weekly during treatment and again at 7 days after the last course of treatment. For each experiment a single observer made all measurements in order to avoid variation in caliper measurements from individual to individual. By convention, partial tumor regression is defined as a reduction in tumor volume of 50% or greater compared to the tumor volume at the time of initiation of treatment. The partial regression rate obtained from a particular treatment is expressed as a percentage; i.e., Number of partial regressions per group/Total number of animals per group x 100. Complete tumor regression was defined as the inability to detect tumor by palpation at the initial site of tumor appearance.

Statistical Evaluation

Differences in the number of partial tumor regressions between treatment groups were compared for statistical significance by chi-square analysis. Differences between groups with $p = 0.05$, or less, were considered significant.

Chemotherapeutic Effects of the Triple Drug Combination and Adria, Alone and in Quadruple Combination (PALA+MMPR+6-AN+Adria) on Spontaneous, Autochthonous CD_8F_1 Breast Tumors

The PALA + MMPR + 6-AN regimen, with (Group 2) or without (Group 1) Adria, was administered at 10-11 day intervals in a series of three separate experiments to a total of 44 CD8F1 mice bearing advanced, spontaneous, autochthonous breast tumors averaging 304 mgs in each group. In each of these experiments, a comparable group of mice was treated with Adria alone at 11 mg/kg which had been determined previously to be the MTD of Adria alone when administered in this 10-11 day treatment schedule. Results were observed 7 days after the third course of treatment.

Table 6

Therapeutic Comparison of PALA + MMPR + 6-AN Chemotherapy With and Without The Addition of Adriamycin (Adria) in CD8F1 Mice Bearing Spontaneous, Autochthonous Breast Cancers[a]

	Treatment[b]	Percent Body Weight Change	Dead/ Total	Partial Tumor Regressions/ Survivors
1.	$PALA_{100}$ -17 hr-> $MMPR_{150}$ + $6\text{-}AN_{10}$	-14	0/42	32/42 (76%)
2.	$PALA_{100}$ -17 hr-> $MMPR_{150}$ + $6\text{-}AN_{10}$ -2½ hr-> $Adria_{6}$	-16	2/44	42/42 (100%)
3.	$Adria_{11}$[c]	-11	1/44	7/43 (16%)

[a] Pooled data: Experiments R559, R560, R561. Tumor weight averaged 304 mgs at the time of initiation of treatment.

[b] Three courses of the indicated treatment were administered with a 10 or 11 day interval between courses. Subscripts refer to doses in mg/kg. Adria was administered i.v. and all other drugs were administered i.p. Observations were recorded 7 days after the third course of treatment.

[c] Adria at 11 mg/kg is the MTD of Adria alone in a 10-11 day treatment schedule.

The PALA + MMPR + 6-AN regimen (Group 1) produced a partial tumor regression rate of 76% in mice bearing spontaneous, autochthonous breast tumors (32 partial tumor regressions in 42 surviving mice with a range of 50-92% in the individual experiments) with no toxic deaths in the treated mice. The addition of Adria to the three-drug regimen (Group 2) resulted in 42 partial tumor regressions in the 42 surviving mice, or 100% (i.e., with no range in the individual experiments), without a significant increase in the mortality rate (only 5%), and with only a 16% body weight loss. The addition of Adria to the PALA, MMPR, 6-AN regimen was found to result in a statistically significant increase in tumor regressions ($p < 0.01$) in mice bearing spontaneous, autochthonous breast tumors. Moreover, 5 of the 42 tumor regressions in Group 2 were complete as opposed to partial regressions, and it should be noted that complete regressions were not observed in the other two groups. It should be noted that Adria alone at 11 mg/kg produced only 16% regressions in 42 treated tumors, and spontaneous regressions of these tumors has not been observed.

A critically important feature of the results of these experiments in mice bearing spontaneous, autochthonous breast tumors can be seen in Figure 1 where the percent of tumors in regression in each of the treatment groups is plotted at 7 days after each of the 3 courses of treatment. Note the diminishing therapeutic effect in mice treated with Adria alone at it's MTD of 11 mg/kg every 10-11 days. After the first course, 43% of the tumors had regressed to 50% or less of their initial size. However, 7 days after the second course, only 21% were partially regressed, and 7 days after the third course, only 16% were still in partial regression. In contrast, in mice treated with PALA + MMPR + 6-AN followed by Adria at 6 mg/kg, the regression rate was 66% after the first course, and then it increased to 93% after the

second course and to 100% after the third course.. Note that none of the tumors in the 42 mice surviving three courses of treatment escaped from the regression-inducing activity of the quadruple-drug regimen. This is an unprecedented response rate in this spontaneous, and highly heterogeneous tumor model, and because of the high degree of chemotherapeutic correlation that has been observed between this model and the human disease we believe that this 4-drug regimen may be a therapeutic breakthrough.

Example 3: PALA, MMPR, 6-AN and Taxol

Murine Breast Tumor System

CD8F1 hybrid mice bearing single spontaneous, autochthonous breast tumors arising during the preceding week were selected from a colony (Stolfi et al., Cancer Chemother. Rep. 55:239-251 (1971) and Martin et al., Cancer Chemother. Rep., Part 2, 5:89-109 (1975)). For each experiment, a tumor cell brei, prepared by pooling 3-4 spontaneously arising CD8F1 breast tumors, was transplanted into syngeneic three-month old mice. In approximately three to four weeks, when transplanted tumors were measurable, the tumor-bearing mice were distributed among experimental groups so that mice carrying tumors of approximately equal weight were represented in each treatment group. Therapy was begun when the tumors were advanced and relatively large; the average tumor weight was close to 130 mg at the beginning of treatment.

As in all spontaneous tumors, whether human or murine, each individual cancer has a heterogeneous cell population. The first generation transplants of CD8F1 breast tumors are obtained from a tumor cell brei made by pooling 3-4 spontaneously arising tumors. Thus, the individual transplants in each experiment develop from a

single brei that, although common to all the mice in that experiments, has a neoplastic cell composition that is likely slightly different from that in another experiment. Therefore, quantitative measurement of any individual parameter (e.g., average tumor size) may be somewhat different from experiment to experiment, but the findings will be quantitatively relevant within individual experiments, as will similar trends among experiments. The CD8F1 first generation breast tumor is included in the murine tumor testing panel of the National Cancer Drug Screening Program (Goldin et al., Eur. J. Cancer 17:129-142 (1981)).

Tumor Measurement

Two axes of the tumor (the longest axis, L and the shortest axis, W) were measured with the aid of a Vernier caliper. Tumor weight was estimated according to the formula: tumor weight (mg) = L (mm) x $(W(mm)^2)/2$.

Chemotherapeutic Agents

MMPR and 6-AN were obtained from Sigma Chemical Co., St. Louis, MO. Each of these agents was dissolved in 0.85% NaCl solution immediately before use. PALA and taxol were obtained from the Department of Health, Education, and Welfare, USPHS of the National Cancer Institute, Bethesda, MD. PALA was dissolved in 0.85% NaCl solution, and the pH was adjusted to 7.2 to 7.5 with 1N NaOH before adjustment to final volume. Taxol was received already soluabilizxed in polyoxyethylated castor oil and dehydrated alcohol. Because of the known toxicity of this diluent, the Taxol stock was diluted, depending upon the dose to be administered, a minimum of 6-fold in saline before injection. For doses below 10 mg/kg, taxol was administered in 0.1 ml/10 g of bodyweight. For doses above 10 mg/kg, an appropriate additional volume was administered. All other agents were administered so that the desired dose was contained in 0.1 ml/10 g of mouse body weight, with the exception of taxol.

These drugs were administered in a timed sequence, with PALA administered 17 hours before MMPR + 6-AN, and taxol administered 2 1/2 hours after MMPR + 6-AN. In one experiment (Exp. 2536, Table 8), Taxol was administered in a fractionated schedule as follows: Taxol (4 mg/kg) simultaneously with MMPR + 6-AN then, 1 1/2 hours later, Taxol (4 mg/kg) q 3 hours x 7.

Determination of Chemotherapy-Induced Tumor Regression Rate

The initial size of each tumor in each treatment group was recorded prior to the initiation of treatment. Tumor size was recorded weekly during treatment and again at 7 days after the last course of treatment. For each experiment a single observer made all measurements in order to avoid variation in caliper measurements from individual to individual. By convention, partial tumor regression is defined as a reduction in tumor volume of 50% or greater compared to the tumor volume at the time of initiation of treatment. The partial regression rate obtained from a particular treatment is expressed as a percentage; i.e., Number of partial regressions per group/total number of animals per group x 100.

Statistical Evaluation

Differences in the size of tumors between treatment groups were compared for statistical significance by the Student's t-test. Differences between groups with $p = 0.05$, or less, were considered significant.

Chemotherapeutic Effect of Taxol Alone In the Treatment of First Passage CD8F1 Murine Advanced Breast Tumors

Table 7 reports a series of four experiments in CD8F1 mice bearing first passage spontaneous CD8F1 advanced breast tumor transplants. Each individual experiment compared a group of saline-treated controls with a second group that received the maximal tolerated dose (MTD) of

taxol alone (80 mg/kg) in a q 10-11 day administration schedule for a total of 3 courses, and observations were recorded 6 days after the last course of treatment.

TABLE 7 ACTIVITY OF TAXOL ALONE IN THE TREATMENT OF FIRST PASSAGE CD8F1 MURINE ADVANCED[a] BREAST TUMORS

Exp. & Group	Treatment[b]	% Body Wt. Change	Dead/Total (%)	Tumor Wt. (mg)
2540				
1.	Saline	-5	9/10 (90%)	7,159
2.	$Taxol_{80}$	-5	0/10 (0%)	1,749
2542				
1.	Saline	+16	9/10 (90%)	10,206
2.	$Taxol_{80}$	- 3	0/10 (0%)	556
2544				
1.	Saline	- 5	5/10 (50%)	4,152
2.	$Taxol_{80}$	- 6	2/10 (20%)	1,140
2546				
1.	Saline	+12	9/10 (90%)	9,982
2.	$Taxol_{80}$	- 7	0/10 (0%)	849

[a] Advanced: Tumor weight averaged 130 (Exp. 2540), 80 (Exp. 2542), 115 (Exp. 2544), and 155 (Exp. 2546) mg. at the time of initiation of treatment.

[b] Three courses of the indicated treatment (i.p) were administered with a 10-11 day interval between courses. Observations were recorded 6 days after the third course of treatment. Subscript is dose at 80mg/kg, the maximum tolerated dose of Taxol on the 10-11 day schedule.

At the end of the observation period, the untreated (saline-treated) controls averaged an 80% mortality due to unrestricted tumor growth. In contrast, the four taxol-treated groups evidenced little toxicity (average weight loss = 5%; average mortality = 5%), and there was marked inhibition of tumor growth. Taxol alone clearly has strong anti-tumor activity in the CD8F1 breast tumor model.

Chemotherapeutic Effects of the Addition of Taxol to the Triple Combination of PALA + MMPR + 6-AN + Taxol In First Passage CD8F1 Advanced Breast Tumors

The triple drug regimen, with (Group 2) or without taxol (Group 1), was administered at 10-11 day intervals in a series of three separate experiments, and observations were recorded 6 days after the third course of treatment.

TABLE 8

ENHANCED ACTIVITY OF TAXOL WHEN ADMINISTERED WITH THE TRIPLE COMBINATION OF PALA + 6-METHYLMERCAPTOPURINE (MMPR) + 6-AMINONICOTINAMIDE (6-AN) IN THE TREATMENT OF FIRST PASSAGE CDF1 MURINE ADVANCED[a] BREAST TUMORS

Exp. + Group	TREATMENT[b]	Percent Body Weight Change	Dead/Total (%)	Tumor Wt. (mg.)
2536				
1.	$PALA_{100}$-17 hr->$MMPR_{150}$ + $6\text{-}AN_{10}$	-21	0/10 (0%)	1301
2.	$PALA_{100}$-17 hr->$MMPR_{150}$ + $6\text{-}AN_{10}$ + $Taxol_4$ -1.5hr-> $Taxol_4$ q3hr x 7	-25	0/10 (0%)	472*
2537				
1.	$PALA_{100}$-17 hr->$MMPR_{150}$	-23	0/10 (0%)	958
2.	$PALA_{100}$-17 hr->$MMPR_{150}$ + $6\text{-}AN_{10}$ -2.5 hr-> $Taxol_{30}$	-23	2/10 (20%)	319*
2539				
1.	$PALA_{100}$-17 hr->$MMPR_{150}$ + $6\text{-}AN_{10}$	-22	1/9 (11%)	271
	$PALA_{100}$-17 hr->$MMPR_{150}$ + $6\text{-}AN_{10}$ -2.5 hr-> $Taxol_{30}$	-21	0/9 (0%)	138[c]

[a] Advanced: Tumor weight averaged 160 (Exps. 2536, 2537) and 87 mg. (Exp. 2539) at the time of initiation of treatment.

[b] Three courses of the indicated treatment (i.p.) were administered with a 10-11 day interval between courses. Observations were recorded 6 days after the third course of treatment.

* $p < 0.05$ (Exp. 2536) or < 0.01 (Exp. 2537) versus group 1.

[c] NS vs group 1 (Exp. 2539), but group 2 had 5/9 (55%) partial tumor regressions compared to only 1/9 (11%) in group 1.

Pooling the toxicity data of the three groups receiving only the triple drug regimen, there was an average weight loss of 22% and a mortality rate of only 3%. In one of the experiments (Exp. 2536), Taxol was added to the triple regimen in the indicated fractionated schedule (Group 2). The weight loss (25%) and absent mortality (0%) in the taxol-containing four drug combination, Group 2, were identical to its three-drug-treated control without taxol, Group 1, but the therapeutic activity was significantly better ($p < 0.05$) than that achieved by the three-drug combination without Taxol.

In two of the experiments (Exp. 2537 and 2539), a single bolus dose of taxol was added to the three-drug combination (Group 2). The pooled toxicity data in these two taxol-containing groups (Group 2) averaged 22% weight loss and 11% mortality, essentially no different than the average of their two three-drug control groups (-23% weight loss; 5% mortality). The therapeutic activity of the taxol-containing combination (Group 2) in Exp. 2537 was significantly better ($p < 0.01$) than that of its three-drug control group without taxol, Group 1. In Exp. 2539, the average tumor weight (138 mg) of the three-drug regimen + Taxol, Group 2, although much smaller than the average tumor weight (271 mg) of its 3-drug control without taxol, Group 1, nevertheless was not significantly different from that of its control (Group 1). But there were 5/9 partial tumor regressions, or a 55% PR, compared to only 1/9 (11%PR) in its three-drug control, Group 1.

The data of Table 8 indicate that the level of therapeutic activity achieved by the quadruple drug combination containing taxol was significantly better in all three experiments than that in the triple drug combination without taxol, and that this improvement in anti-tumor activity was achieved without increase in toxicity.

Chemotherapeutic Effects of the Triple-Drug Combination and Taxol at MTD, Alone and in Quadruple Combination (PALA + MMPR + 6-AN + Taxol), In First Passage CD8F1 Murine Advanced Breast Tumors

Two experiments are presented in Table 9, each consisting of four groups of ten tumor-bearing animals each, as follows: Group 1, saline-treated controls; Group 2, Taxol at 80 mg/kg; Group 3, PALA + MMPR + 6-AN; and Group 4, PALA + MMPR + 6-AN + Taxol at 25 mg/kg. Treatment was repeated at 10 or 11 day intervals in all groups for a total of 3 courses, and observations were recorded 6 days after the last course of treatment.

TABLE 9 THERAPEUTIC ACTIVITY OF TAXOL ALONE AT MTD COMPARED TO TAXOL ADDED TO THE PALA + 6-MERCAPTOPURINE RIBOSIDE (MMPR) + 6-AMINONICOTINAMIDE (6-AN) COMBINATION IN THE TREATMENT OF FIRST PASSAGE (CD8F1) MURINE ADVANCED[a] BREAST TUMORS

Exp. & Group	Treatment[b]	% Body Wt. Change	Dead/Total (%)	Tumor Wt. (mg)
2542				
1.	Saline	+16	9/10 (90%)	10,206
2.	$Taxol_{80}$	- 3	0/10 (0%)	556[c]
3.	$PALA_{100}$-17hr-$MMPR_{150}$+6-AN_{10}	-11	2/10 (20%)	859[c]
4.	$PALA_{100}$-17hr-$MMPR_{150}$+6-AN_{10} -2.5hr-$Taxol_{25}$	-13	0/10 (0%)	200*
2544				
1.	Saline	- 5	5/10 (50%)	4,152
2.	$Taxol_{80}$	- 6	2/10 (20%)	1,140*
3.	$PALA_{100}$-17hr-$MMPR_{150}$+6-AN_{10}	-15	0/10 (0%)	312**
4.	$PALA_{100}$-17hr-$MMPR_{150}$+6-AN_{10} -2.5hr-$Taxol_{25}$	-20	0/10 (0%)	63[d]

[a] Advanced: Tumor weight averaged 80 (Exp. 2542) and 115 mg (Exp. 2544) at the time of initiation of treatment.

[b] Three courses of the indicated treatment (i.p.) were administered with 10-11 day interval between courses. Observations were recorded 6 days after the third course of treatment.

[c] Groups 2 and 3 (Exp. 2542) cannot be statistically compared to group 1, Saline controls, where only 1 tumor-bearing mouse is alive. 90% mortality due to untreated tumor growth.

* $p<0.05$, when group 4 is compared to groups 2 & 3 (Exp. 2542), and when group 2 is compared to group 1 (Exp. 2544).

** $p<0.001$ when group 3 is compared to group 1 (Exp. 2544).

[d] In Exp. 2544, group 4 has a 40% partial tumor regression rate, whereas there are no partial tumor regressions in the other groups.

In Exp. 2542, Group 1, saline, had a 90% mortality due to the unrestricted growth of untreated tumors. Therefore, the average tumor size in Group 2, $Taxol_{80}$ alone, and in Group 3, the triple combination, cannot be compared statistically to the single tumor-bearing mouse in Group 1, saline. However, from the differences in tumor-induced mortality rates between these three groups, it is clear that the tumors of groups 2 and 3 were markedly inhibited by their respective treatments. Group 4, the taxol-containing four drug combination, had tumors that were significantly inhibited compared to the tumors in the three drug combination without taxol, Group 3, and to taxol alone, Group 2, and this was achieved with little toxicity (13% weight loss; 0% mortality). It should be noted that the superior anti-tumor activity of Group 4 was achieved with a dose of taxol (25 mg/kg) that was less than one-third that of taxol alone (80 mg/kg), Group 2.

In Exp. 2544, taxol alone at 80 mg/kg (Group 2), and the triple drug combination (Group 3), significantly inhibited tumor growth over that of the saline-treated tumors (Group 1). The anti=tumor activity of the three-drug combination with taxol (Group 4) is clearly superior to all other groups because a 40% PR rate was induced (see d, legend, Table 9), and there were no partial tumor regressions produced in any of the other groups. Again, it should be noted that the superior anti-tumor activity by the taxol-containing quadruple combination, Group 4, was achieved with a dose of taxol (25 mg/kg) approximately one-third that of the MTD dose (80 mg/kg) of Taxol alone, Group 2, and this increase in anti-tumor activity was achieved without mortality.

Example IV: PALA, MMPR, 6-AN and Radiation

The PALA-MMPR-6-AN combination also sensitizes tumors to ionizing radiation therapy.

Mice with advanced transplanted CD8F1 breast tumors (initial tumor weight 150mg) were divided into four treatment groups:

1) Saline Control
2) PALA-MMPR-6-AN
3) PALA-MMPR-6-AN + radiation (15 Gy, localized)
4) Radiation (15 Gy, localized)

Three courses of these treatments were administered, with a 10-11 day interval between courses. Results and details of treatment timing and drug doses are indicated in Table 10.

While radiation therapy alone (group 4) significantly improved survival and retarded tumor growth, no tumor regressions were observed. In contrast, when mice were treated before irradiation with PALA-MMPR-6-AN, 8 out of 10 mice had regressions (tumor size less than 50% of original weight), and 3 of those regressions were complete.

* * *

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

TABLE 10

Therapy of Advanced First Passage CD8F1 Breast Tumors with PALA + HMPR + 6-AN
In Conjunction with Radiation

Regressions/ Treatment[a]	Percent Body Weight Change	Dead/ Total	Tumor W;t (mgs)	Survivors
1. Saline Control	-22	5/10	6545	0/5
2. $PALA_{100}$-17hr-> $^{HMPR_{150}}_{6\text{-}AN_{10}}$	-34	8/10	5170	0/2
3. $PALA_{100}$-17hr-> $^{HMPR_{150}}_{6\text{-}AN_{10}}$ -10hr-> Radiation	- 2	0/10	54**	5PR + 3CR/ 10
4. Radiation	0	0/10	424++	0/10

[a] Exp. 2525H; tumor w't. = 150 mg when treatment initiated. Three courses of the indicated treatment were administered with a 10-11 day interval between courses. Subscripts refer to doses in mg/kg. Radiation was administered at 15 Gy. Observations are recorded at 36 days after the third course of treatment.

** $p < 0.01$ when compared to group 1.
++ $p < 0.01$ when compared to group 3.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising:
aa) inhibitor of purine nucleotide biosynthesis, and
b) a nicotinamide antagonist.

2. A pharmaceutical composition as in claim 1 wherein said inhibitor of purine nucleotide biosynthesis is selected from the group consisting of MMPR, 6-mercaptopurine, thioguanine, thiamiprine, tiazofurin, azaserine, 6-diazo-5-oxo L-norleucine, methotrexate, trimetrexate, pteropterin, denopterin and DDATHF.

3. A pharmaceutical composition as in claim 1 wherein said nicotinamide antagonist is selected from the group consisting of 6-AN, thionicotinamide, 2-amino-1,3,4-thiadiazole, 2-ethylamino-1,3,4-thiadiazole, 6-aminonicotinic acid, 5-methylnicotinamide and 3-acetylpyridine.

4. A pharmaceutical composition as in claim 1 further comprising a pharmaceutically acceptable carrier.

5. A pharmaceutical composition as in claim 1 comprising:
a) MMPR, and
b) 6-AN.

6. A kit comprising:
a) a vial containing an inhibitor of purine biosynthesis and a niacin antagonist, and
b) a vial containing an apoptosis inducing agent.

7. A kit comprising:
a) a vial containing an inhibitor of purine biosynthesis,

b) a vial containing a nicotinamide antagonist, and
c) a vial containing an apoptosis inducing agent.

8. A kit as in claim 6 or 7 further comprising a vial containing an inhibitor of pyrimidine biosynthesis.

9. A kit as in claim 6 or 7 wherein said inhibitor of purine biosynthesis is selected from the group consisting of MMPR, 6-mercaptopurine, thioguanine, thiamiprine, tiazofurin, azaserine, 6-diazo-5-oxo L-norleucine, methotrexate, trimetrexate, pteropterin, denopterin and DDATHF.

10. A kit as in claim 6 or 7 wherein said nicotinamide antagonist is selected from the group consisting of 6-AN, thionicotinamide, 2-amino-1,3,4-thiadiazole, 2-ethylamino-1,3,4-thiadiazole, 6-aminonicotinic acid, 5-methylnicotinamide and 3-acetylpyridine.

11. A kit as in claim 6 or 7 wherein said apoptosis inducing agent is selected from the group consisting of methotrexate, 5-fluorodeoxyuridine, 5-fluorouracil, 1-B-D-arabinofuranosyl- cytosine, puromycin, trifluorothymidine, cisplatin, etoposide, camptothecin, cytoxan, adriamycin, teniposide, podophyllotoxin, aphidocolin, sodium azide, N-methyl-N'-nitro-N'nitrosoguanidine, nitrogen mustard, bleomycin, 1,3-bis(2-chloroethyl)-a-nitrosourea, methyl glyoxal-bis-(guanylhydrazone), colcemid, vincristine, taxol, taxotere, dexamethasone, retinoic acid, purinergic P2 receptor agonists, somatostatin analogs, luteinizing hormone releasing factor analogs, and antibodies capable of inducing apoptosis.

12. A kit as in claim 8 wherein said inhibitor of pyrimidine biosynthesis is selected from the group

consisting of PALA, 6-azauridine, triacetyl-6-azauridine, pyrazofuran, brequinar and acivicin.

13. A method of treating antineoplastic disease in an animal comprising the steps of:

a) administering a therapeutically effective amount of an inhibitor of purine biosynthesis,

b) administering a nicotinamide antagonist, and

c) administering an apoptosis inducing agent.

14. A method as in claim 13 further comprising the step of d) administering a therapeutically effective amount of an inhibitor of pyrimidine biosynthesis.

15. A method as in claim 13 wherein said inhibitor of purine biosynthesis is selected from the group consisting of MMPR, 6-mercaptopurine, thioguanine, thiamiprine, tiazofurin, azasevine, 6-diazo-5-oxo L-norleucine, methotrexate, trimetrexate, pteropterin, denopterin and DDATHF.

16. A method as in claim 13 wherein said nicotinamide antagonist is selected from the group consisting of 6-AN, thionicotinamide, 2-amino-1,3,4-thiadiazole, 2-ethylamino-1,3,4-thiadiazole, 6-aminonicotinic acid, 5-methylnicotinamide and 3-acetylpyridine.

17. A method as in claim 13 wherein said apoptosis inducing agent is selected from the group consisting of methotrexate, 5-fluorodeoxyuridine, 5-fluorouracil, 1-B-D-arabinofuranosyl- cytosine, puromycin, trifluorothymidine, cisplatin, etoposide, camptothecin, cytoxan, adriamycin, teniposide, podophyllotoxin, aphidocolin, sodium azide, N-methyl-N'-nitro-N'hitrosoguanidine, nitrogen mustard, bleomycin, 1,3-bis(2-chloroethyl)-a-nitrosourea, methyl glyoxal-bis-(guanylhydrazone), colcemid, vincristine,

taxol, taxotere, dexamethasone, retinoic acid, purinergic P2 receptor agonists, somatostatin analogs, luteinizing hormone releasing factor analogs, antibodies capable of inducing apoptosis, and cytotoxic T-cells.

18. A method as in claim 14 wherein said inhibitor of pyrimidine biosynthesis is selected from the group consisting of PALA, 6-azauridine, triacetyl-6-azauridine, pyrazofuran, brequinar and acivicin.

19. A method as in claim 13 wherein step c) occurs after steps a) and b).

20. A method as in claim 14 wherein said administering a therapeutically effective amount of an inhibitory of pyrimidine biosynthesis step occurs before steps a), b) and c).

21. A method as in claim 13 wherein said inhibitor of purine biosynthesis is MMPR, said nicotinamide antagonist is 6-AN, and said apoptosis inducing agent is FUra.

22. A method as in claim 13 wherein said inhibitor of purine biosynthesis is MMPR, said micotinamide antagonist is 6-AN, and said apoptosis inducing agent is FUra.

23. A method as in claim 13 wherein said inhibitor of purine biosynthesis is MMPR, said micotinamide antagonist is 6-AN, and said apoptosis inducing agent is taxol.

24. A method of treating antineoplastic disease in an animal comprising the steps of:

a) administering a therapeutically effective amount of an inhibitor of purine biosynthesis,

b) administering a nicotinamide antagonist, and

c) administering radiotherapy.

25. A method as in claim 24 further comprising the step of administering a therapeutically effective amount of an inhibitor of pyrimidine biosynthesis.

26. A method of treating multiple drug resistance in an animal comprising the steps of:

a) administering a therapeutically effective amount of an inhibitor of purine biosynthesis,

b) administering a nicotinamide antagonist, and

c) administering an apoptosis inducing agent.

27. A method as in claim 26 further comprising the step of administering a therapeutically effective amount of an inhibitor pyrimidine biosynthesis.

Fig. 1

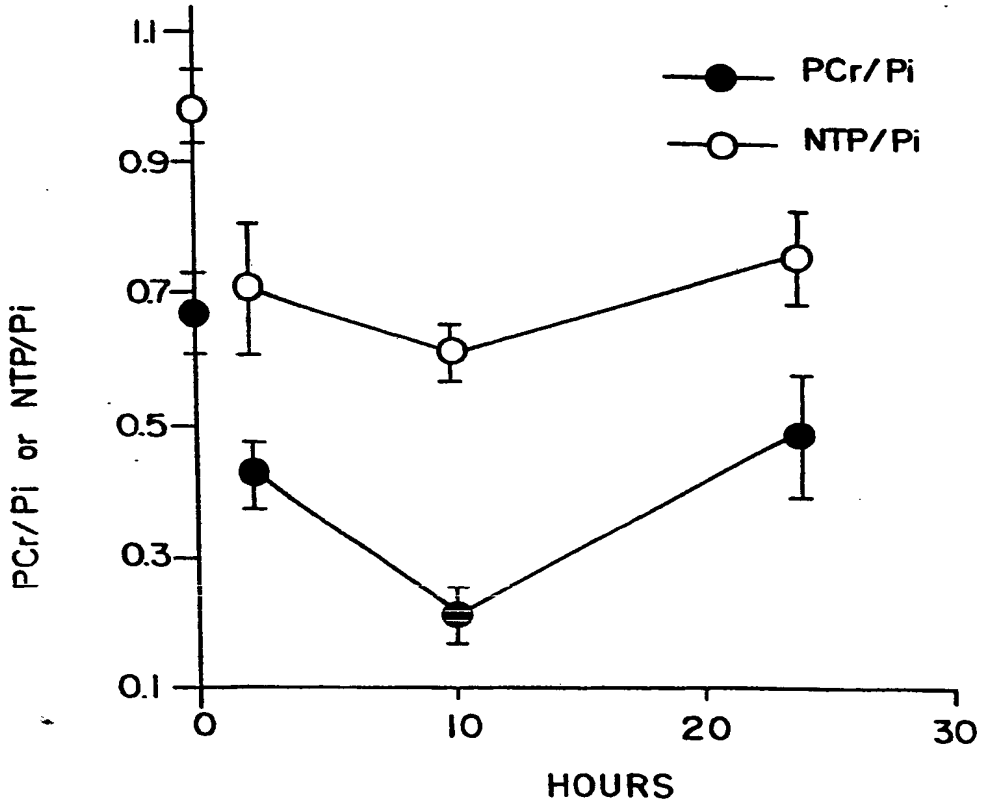
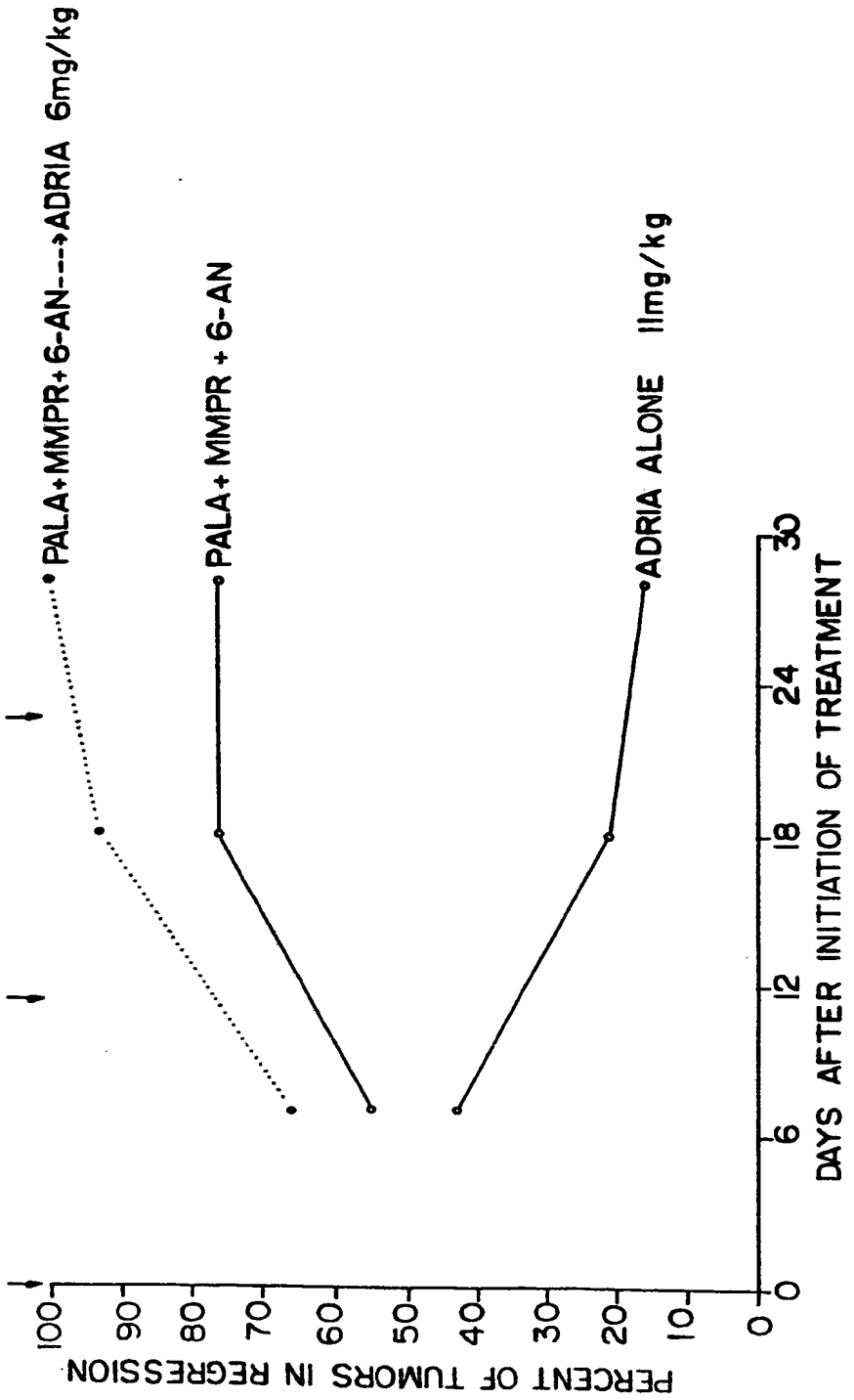


Fig. 2


PALA+MMPR+6-AN---→ADRIA 6mg/kg
PALA+MMPR+6-AN
ADRIA ALONE 11mg/kg
PERCENT OF TUMORS IN REGRESSION
0
10
20
30
40
50
60
70
80
90
100
DAYS AFTER INITIATION OF TREATMENT
0
6
12
18
24
30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04775

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 9/08
US CL :424/423

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/423

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,114,951 (KING) 19 MAY 1992, SEE ENTIRE DOCUMENT.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 14 JULY 1993	Date of mailing of the international search report 09 SEP 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer EDWARD J. WEBMAN Telephone No. (703) 308-2351

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